UNIVERSIDAD AUTONOMA DE MADRID

EVALUACIÓN DE LAS PROPIEDADES BIOLÓGICAS DE ANTIOXIDANTES FENÓLICOS EN SUBPRODUCTO DE UVA, CAFÉ VERDE Y YERBA MATE COMO INGREDIENTES FUNCIONALES O NUTRACÉUTICOS

EVALUATION OF BIOLOGICAL PROPERTIES OF PHENOLIC ANTIOXIDANTS IN GRAPE POMACE, GREEN COFFEE AND YERBA MATE AS FUNCTIONAL INGREDIENTS OR NUTRACEUTICALS

Tesis Doctoral/ Doctoral Thesis

Shenli Wang

Madrid, 2016

CSIC
Consejo Superior de Investigaciones Científicas

ictaN
INSTITUTO DE CIENCIA, TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN

UNIVERSIDAD AUTÓNOMA DE MADRID

AUTONÓMA UNIVERSITY OF MADRID

FACULTAD DE CIENCIAS

FACULTY OF SCIENCES

EVALUACIÓN DE LAS PROPIEDADES BIOLÓGICAS DE ANTIOXIDANTES FENÓLICOS EN SUBPRODUCTO DE UVA, CAFÉ VERDE Y YERBA MATE COMO INGREDIENTES FUNCIONALES O NUTRACÉUTICOS

EVALUATION OF BIOLOGICAL PROPERTIES OF PHENOLIC ANTIOXIDANTS IN GRAPE POMACE, GREEN COFFEE AND YERBA MATE AS FUNCTIONAL INGREDIENTS OR NUTRACEUTICALS

Tesis Doctoral/ Doctoral Thesis

Shenli Wang

Madrid, 2016

Facultad de Ciencias

Doctorado en Biología y Ciencias de la Alimentación

Faculty of Sciences

PhD in Biology and Food Sciences

Instituto de Ciencia y Tecnología de Alimentos y Nutrición

Institute of Food Science, Technology and Nutrition

EVALUACIÓN DE LAS PROPIEDADES BIOLÓGICAS DE ANTIOXIDANTES FENÓLICOS EN SUBPRODUCTO DE UVA, CAFÉ VERDE Y YERBA MATE COMO INGREDIENTES FUNCIONALES O NUTRACÉUTICOS

EVALUATION OF BIOLOGICAL PROPERTIES OF PHENOLIC ANTIOXIDANTS IN GRAPE POMACE, GREEN COFFEE AND YERBA MATE AS FUNCTIONAL INGREDIENTS OR NUTRACEUTICALS

Memoria que presenta Shenli Wang para optar al grado de Doctor por la Universidad Autónoma de Madrid / *PhD dissertation submitted by Shenli Wang to obtain her PhD Degree at the Autonoma University of Madrid*

Realizada bajo la dirección de / *Under the supervision of* Dr. Beatriz Sarriá Ruiz, Dr. Miryam Amigo-Benavent, Prof. Laura Bravo Clemente

INSTITUTO DE CIENCIA Y TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN
INSTITUTE OF FOOD SCIENCE, TECHNOLOGY AND NUTRITION
(ICTAN-CSIC) Madrid, 2016

THIS DOCTORAL THESIS HAS BEEN SUPPORTED BY THE FOLLOWING FUNDING:

ESTA TESIS DOCTORAL HA SIDO REALIZADA GRACIAS A LA FINANCIACION DE DIFERENTES AYUDAS Y PROYECTOS:

- PhD Scholarship Chinese Scholarship Council (Chinese Government, CSC No. 201206350027) from September 2012 to August 2016. Beca predoctoral del Consejo de Educación del Gobierno Chino (sept 2012-agosto 2016).
- The Spanish Ministry of Economy and Competitiveness, R+D+R Spanish National plan projects AGL2010-18269 and AGL2015-69986-R. Los proyectos AGL2010-18269 and AGL2015-69986-R del Plan Estatal de I+D+I, Ministerio de Economía y Competitividad.
- The Ministry of Economy and Competitiveness, R+D+R Consolider-Ingenio CSD2007-00063 and INCOMES. Proyecto Consolider-Ingenio CSD2007-00063 e INCOMES del Plan Estatal de I+D+I, Ministerio de Economía y Competitividad.
- Joint Collaborative Project among R+D Technology research groups in the Community of Madrid S2013/ABI-3028, Community of Madrid (Spain). Proyecto S2013/ABI-3028 del Programa de Actividades de I+D entre grupos de investigación de la Comunidad de Madrid en Tecnologías.
- Spanish National Research Council (CSIC) Project PIE201670E061. Proyecto PIE201670E061 del Consejo Superior de Investigaciones Científicas (CSIC, España).

Acknowledgments

First of all, I would like to express my great gratitude to my thesis supervisors, Prof. **Laura Bravo**, for recruiting me in her research group, for giving me the chance to study in the Institute of Food Science, Technology and Nutrtition (ICTAN), for giving me their foresight, for guiding the PhD project, patience supervision, and corrections for the manuscripts and PhD Thesis. Her perfect and professional attitude to the work sets a good example for me and inspires me to be better in the future work. I am also very grateful to my co-supervisor Dr. **Beatriz Sarriá** for her helpful corrections and advices for my whole Thesis and manuscripts, for her always kindness supports me during the development of the PhD work, for helping me in the experiments. And I am also very grateful for her kindness care on my life and studies in Spain. I am also very grateful to my another co-supervisor Dr. **Miryam Amigo**, for giving me a lot of help when I joined our group, for teaching me all the practical issues of cell experiments, for correcting the manuscripts and helping me with the final Thesis. I learned a lot when worked with her. All praise goes to them if I could make contributions in the future. Without their effort and support, the Thesis could not be finished. Under the supervision of known and knowledgeable scientists has always pushed me to be perfected for a high level of expertise. I have learned a lot from their tirelessly enthusiastic pursuit of scientific questions.

Moreover, I would also like to thank Dr. Raquel Mateos for her very kind help with the results and corrections of manuscripts. I would also like to thank Prof. Luis Goya for his kindness help with the antioxidant enzyme experiments, useful and helpful discussion about the manuscripts, and also for his open-mind, pleasure coffee time brings me good spirits. I would specifically like to thank Dr. Sonia Ramos and Dr. M. Angeles Martín in the BIOCELL group, for helping me a lot when I need help in the experiments.

I would also like to thank people who help me a lot in the ICTAN, thank Francisco Morales for his kindness lending the plate reader, thank Miguel Angel Martin for colorimeter teaching and his care when help was needed, I thank Immaculada Alvarez for Masshunter teaching, and Gema Campos for the microbiologic analysis.

My research progress would not have been possible without the help of all of lab mates. I would also like to thank some other colleagues in the BIOCELL group including Gema Beaza, Isabel Cordero, and Sara Martínez for their kindness care and

help in the work. And I would also like to thank Marta Mesías, Marta Navarro, Jara Pérez, Isabel the receptionist, María Blanch in the ICTAN who are very kind and made my days at institution enjoyable.

Finally I would like to thank my family and my parents whose support is the powerful resource for my academic career, and my friends' encouragement during my studies. I would also like to take this opportunity to memory to my lovely grandma who encouraged me to be brave. Hence, I need to thank for the "China Scholarship Council" for offer me the fellowship to study abroad.

Time flies, I would definitely remember the days and lifestyle here which is a very precious experience and moments in the life.

Table of Contents

List of Figures

List of Tables

Abbreviations

3-CQA: 3-caffeoylquinic acid

3-FQA: 3-feruloylquinic acid

3,4-DCQA: 3,4-dicaffeoylquinic acid

3,5-DCQA: 3,5-dicaffeoylquinic acid

3,5-DCQA: 3,5-dicaffeoylquinic acid

4-CQA: 4-*O*-caffeoylquinic acid

4-FQA: 4-feruloylquinic acid

4,5-DCQA: 4,5-dicaffeoylquinic acid

5-CQA: 5-caffeoylquinic acid

5-FQA: 5-feruloylquinic acid

AAPH: 2,2'-Azobis(2-amidinopropane dihydrochloride)

$ABTS^{\bullet+}$: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid

AC: adenylate cyclase

ACN: acetonitrile

AGEs: advanced glycation end products

AKT: protein kinase B

ANOVA: analysis of variance

AP-1: activator protein-1

AUC: area under the curve

BrdU: bromodeoxyuridine;

CA: caffeic acid

Caco-2: human epithelial colorectal adenocarcinoma cells

c.f.u.: colony forming units

CGAs: chlorogenic acids

Chr. Peak: chromatographic peak

COX: cyclooxygenase

CVD: cardiovascular disease

DAG: diacylglycerol

DCFH: dichlorofluorescein

DCFH-DA: 2',7'-dichlorofluorescin diacetate

d.m.: dry matter

DMSO: dimethyl sulfoxide

DHCA: dihydrocaffeic acid

DHFA: dihydroferulic acid

DMEM: Dulbecco's modified Eagle's medium

DNPH: 2,4-dinitrophenylhydrazone

DTT: dithiothreitol

ECG: epicatechin gallate

EDTA: ethylenediaminetetraacetic acid

EGCG: epigallocatechin gallate

ERKs: extracellular regulated kinases

eNOS: endothelial nitric oxide synthase

FA: ferulic acid

FBS: fetal bovine serum

FM: fresh matter

FRAP: ferric reducing antioxidant power

GA: gallic acid

GAE: gallic acid equivalents

GCBE: green coffee bean extract

GD-RGP: gastric digested red grape pomace

GD-CT: gastric digestion control (with no added pepsin)

GID-RGP: gastrointestinal digested red grape pomace

GID-CT: gastrointestinal digestion control (with no added pancreatin and bile salts)

GP: grape by-product/pomace

GPE: grape pomace phenolic extract

GPx: glutathione peroxidase

GR: glutathione reductase

GSE: grape seed polyphenolic extract

GSH: glutathione (reduced)

GSSG: glutathione disulphide

HepG2: human hepatoma cell line

HDL: high-density lipoprotein

HDL-C: high-density lipoprotein-cholesterol

HIF1α: hypoxia-inducible factor 1α

HPLC: high pressure liquid chromatography

HUVEC: human umbilical vein endothelial cell

IFN-γ: interferón gamma

IL: interleukin

iNOS: inducible nitric oxide synthase

LDH: lactate dehydrogenase

LDL: low-density lipoprotein

LDL-C: low-density lipoprotein-cholesterol

MAPK: p38 mitogen-activated protein kinase

MDA: malondialdehyde

MEM: minimum essential culture medium

MF: molecular formula

MS: mass spectrometry

n.d.: not detected

NEAA: non-essential amino acids

NADPH: nicotinamide adenine dinucleotide phosphate

NADH: nicotinamide adenine dinucleotide

NF-κB: nuclear factor kappa B

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOS: nitric oxide synthase

Nrf2: NF-erythroid 2-related factor

OPT: *o*-phthaldialdehyde

ORAC: oxygen radical antioxidant capacity

PCA: plate count agar

p-JNK: phospho-Jun kinase

PI3K: phosphoinositide-3-kinase

PKA: protein kinase A,

PKC: protein kinase C

PLCγ: phospholipase Cγ

QToF: quadrupole time-of-flight

RGP: red grape pomace

RGPE: red grape pomace phenolic extract

ROS: reactive oxygen species

RT: retention time

S1P: sphingosine-1-phosphate

SA: syringic acid

SDS: sodium dodecyl sulphate

SOD: superoxide dismutase

SRM: selected reaction monitoring

T2DM: type 2 diabetes

t-BOOH: *tert*-butylhydroperoxide

TC: total cholesterol

TE: Trolox equivalents

TG: triglyceride

TNF-α: tumour necrosis factor alpha

Trolox: 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid

TPTZ: 2,4,6-tri-(2-pyridyl)-1,3,5-triazine

VEGF: vascular endothelial growth factor

VRBA: violet red bile agar

WHO: World Health Organization

WST-1: 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt

YME: yerba mate extract

Introduction

2.1 Phenolic compounds

2.1.1. Types and chemical structure of phenolic compounds, presence in foods and estimated intakes

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir et al., 2004). Fruits, vegetables, whole grains as well as other types of foods and beverages such as tea, coffee, chocolate and wine are rich sources of phenols. More than 8000 phenolic structures have been identified (Tsao, 2010), with great diversity of structures that can range from simple molecules (monomers and oligomers), such as phenolic acids, to highly polymerized compounds, such as tannins (Bravo, 1998).

Most foods contain complex mixtures of polyphenols. Polyphenol composition in foods differs among varieties, and other factors such as the time of harvest, environmental factors, processing, storage, extraction methods and so on, affect the polyphenol content of plant foods. Environmental factors include both climatic factors (temperature, sun exposure, rainfall) and agronomic (soil type, culture in greenhouses or fields, biological culture, hydroponic culture, fruit yield per tree, etc.). The degree of ripeness may also considerably affect the concentration of polyphenols; in general terms, phenolic acid concentrations decrease during ripening, whereas anthocyanin concentrations increase (Manach et al., 2004). The total phenolic content separated by groups of foods is shown in Table 1.

Table 1. Polyphenolic content of different plant foods and beverages.

Plant Foods	Polyphenolic Content	Beverages	Polyphenolic Content
Legumes	0.1-1.5 (% d.m.)	Tea	15-30 (% dry leaves)
Cereals	0.1-10 (% d.m.)		150-210 (mg/200 mL)
Vegetables	0.3-10 (% d.m.)	Coffee	6-9 (% dry beans)
Fruits	2-225 (mg/100 g f.m.)		200-550 (mg/200 mL)
Berries	20-400 (mg/100 g f.m.)	Cocoa	12-18 (% dry beans)
Nuts	0.1-34 (% d.m.)	Wine	250-4000 (mg/L)
Olive oil	0.5-8%	Fruit juices	2-7000 (mg/L)
		Beer	60-100 (mg/L)

d.m.: dry matter; f.m.: fresh matter. From Saura-Calixto and Bravo, 2001

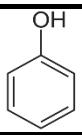
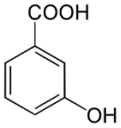
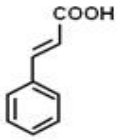
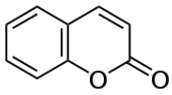
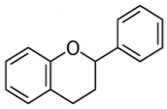
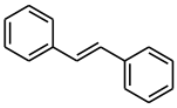
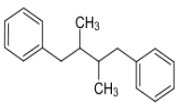
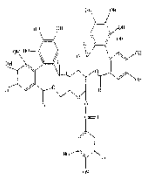
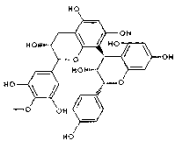
Phenolic compounds have been classified according to their source of origin, biological function and chemical structure. In many cases, these compounds are classified attending to the chemical structure, specifically the number of phenol rings, such as in the Phenol-Explorer database (www.phenol-explorer.eu) (Neveu et al., 2010)) (Table 2).

Phenolic acids

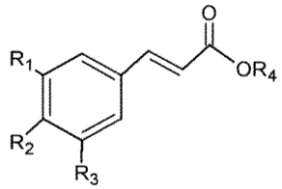
Phenolic acids are low-molecular weight compounds, which can be divided in two main groups: derivatives of benzoic acids based on C1–C6 backbone and derivatives of cinnamic acids which are based on C3–C6. Phenolic acids can only be freed by acid or alkaline hydrolysis or by enzymes (Tsao, 2010).

Benzoic acids (Figure 1) include protocatechuic, vanillic, gallic, and syringic acids. Hydroxybenzoic acids are components of complex structures such as hydrolyzable tannins (gallotannins in mangoes and ellagitannins in red fruits such as pomegranate, strawberries, raspberries, and blackberries). Cinnamic acid (Figure 1) is one of the most representative classes of phenolic acids found in many plants such as grapes, tea, green coffee beans, yerba mate, etc. Hydroxycinnamic acids are more common than hydroxybenzoic acids and consist mainly of *p*-coumaric, caffeic, ferulic, and sinapic acids. These acids are rarely found in the free form, except in processed foods that have undergone freezing, sterilization or fermentation. The bound forms are glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid. Caffeic and quinic acid combine to form chlorogenic acids (Manach et al., 2004).

Table 2. Main classes of phenolic compounds. (Modified from Bravo, 1998)

Class	Subclass	Basic Skeleton	Basic Structure	Examples	Food resource
Simple phenols		C_6		Cresol, Thymol, Resorcinol	Tomato, wheat, oats
Phenolic acid	hydrobenzoic acids	C_6-C_1		Gallic acid, Syringic acid,	Grape pomace, beer, black tea, berries
	hydroxycinnamic acids	C_6-C_3		Caffeic acid, Ferulic acid, *p*-Coumaric acid	Green coffee bean, yerba mate, coffee carrots, potatoes, berries
Coumarins		C_6-C_3		Umbelliferone, Aesculetin, Herniarin, Psoralen, Imperatori	Apricots, strawberries, cinnamon, cherries
Flavonoids		C_6–C_3–C_6		(+)-Catechin, (-)-Epicatechin, Cyanidin, Malvidin, Kaempferol, Quercetin, Apigenin, Daidzein, Eriodictyol	Apples, berries, cocoa, red grapes, wine, teas, onions, yerba mate, soybean, olive oil
Stibenes		C_6-C_2-C_6		Resveratrol, Astringin	Grape, red wine
Lignans		$(C_6$-$C_3)_2$		Secoisolariciresinol, Matairesinol, Pinoresinol	Linseed, whole grains, sesame seeds, flaxseed, red wine
Tannins	hydrolysable tannin	$(C_6$-$C_3)_n$		Ellagitannins	Berries, beer, walnuts
	condensed tannins	$(C_6$-$C_3)_n$		Procyanidins B1, B2,C1	Berries, teas, coffee, pomegranates

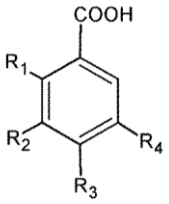
A



Cinnamic acid derivatives

Cinnamic acids derivatives	R_1	R_2	R_3	R_4
Caffeic acid	OH	OH	H	H
Ferullic acid	OCH_3	OH	H	H
Sinapic acid	OCH_3	OH	OCH_3	H
p-Coumaric acid	H	OH	H	H
Chlorogenic acid	H	OH	OH	$C_7H_9O_3$

B



Benzoic acid derivatives

Benzoic acids derivatives	R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH
Syringic acid	H	OCH_3	OH	OCH_3
Vanillic acid	H	H	OH	OCH_3
Protocatechuic acid	H	H	OH	OH
p-Hydroxybenzoic acid	H	H	OH	H

Figure 1. Benzoic and cinnamic acids in foods. Structures of cinnamic acid derivatives (A) and benzoic acids (B).

Flavonoids

Flavonoids are the most common and widely distributed group of phenolic compounds; more than 4000 compounds have been identified according to Harborne and Baxter (1999). Their common backbone structure is C6-C3-C6 in which the two C6 units (rings A and B) are of phenolic nature (Figure 2). Attending to the hydroxylation pattern and variations in the chromane ring (ring C), flavonoids can be further divided into different sub-groups such as anthocyanins, flavanols, flavones, flavanones, flavonols and isoflavones (Figure 3).

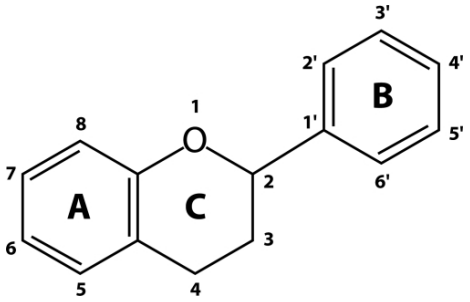


Figure 2. Basic structure of flavonoids

Flavonols are the most ubiquitous flavonoids in foods, being quercetin and kaempferol the main representatives (Figure 3A). They are generally present at relatively low concentrations (15–30 mg/kg fresh weight), in glycosylated forms, being the associated sugar moiety often glucose or rhamnose, but other sugars may also be involved, such as galactose, arabinose, etc. Onions are the richest source (up to 1.2 g/ kg fresh weight), followed by curly kale, leeks, broccoli, and blueberries Red wine and tea may contain up to 45 mg flavonols/L (Manach et al., 2004). The biosynthesis of these compounds is stimulated by light.

Flavones are much less common than flavonols in fruits and vegetables, and consist predominantly of glycosides of luteolin and apigenin (Figure 3B). The only important edible sources of flavones identified to date are parsley and celery. Cereals such as millet and wheat contain C-glycosides of flavones (Feng et al., 1988).

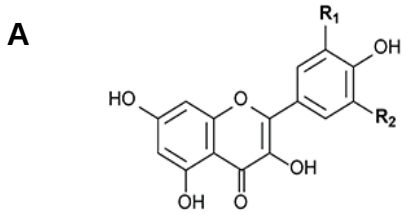
Flavanones are present in high concentrations only in citrus fruits. The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons (Figure 3C). Flavanones are generally glycosylated by a disaccharide at position 7: either a neohesperidose, which imparts a bitter taste (such as to naringin in grapefruit), or a rutinose, which is flavorless. Orange juice contains between 200 and 600 mg hesperidin/L and 15–85 mg narirutin/L, and a single glass of orange juice may contain between 40 and 140 mg flavanone glycosides (Tomás-Barberán and Clifford, 2000).

Isoflavones are flavonoids with structural similarities to oestrogens and thus are classified as phytoestrogens (Figure 3D). The hydroxyl groups in positions 7 and 4' result in a configuration is analogous to that of the hydroxyls in

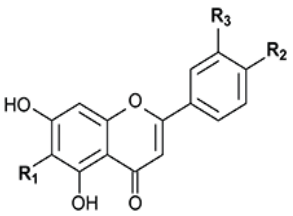
the estradiol molecule and confers pseudohormonal properties to these phenols, including the ability to bind to oestrogen receptors. Isoflavones are found almost exclusively in leguminous plants. Soy and derivated products, which contain three main molecules: genistein, daidzein, and glycitein, generally in a concentration ratio of 1:1:0.2, are the main source of isoflavones in the human diet (Manach et al., 2004).

Flavanols (Figure 3E) are present in both the monomer (catechins) and polymer form (proanthocyanidins). Catechins are found in many types of fruits (apricots, which contain 250 mg/kg fresh wt, are the richest source). They are also present in red wine (up to 300 mg/L), but green tea and chocolate are by far the richest sources. Catechin and epicatechin are the main flavanols in fruits, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Arts et al., 2000a; Arts et al., 2000b). In contrast to other classes of flavonoids, flavanols are not glycosylated in foods. Proanthocyanidins are the major polyphenols in grapes, mostly present in skins and seeds. Seed proanthocyanidins are partly galloylated procyanidins, with degrees of polymerisation in the range of 1 (monomers) to 20 (Prieur et al., 1994). Skin proanthocyanidins contain both procyanidin and prodelphinidin units and are much larger than skin tannins (about 30 units, on average) (Souquet et al., 1996). In addition, proanthocyanidins (condensed tannins) are responsible for the astringent character of fruits (grapes, peaches, kakis, apples, pears, berries, etc.) and beverages (wine, cider, tea, beer, etc.) and for the bitterness of chocolate.

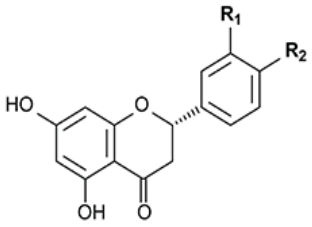
Anthocyanidins are the most important group of water soluble flavonoids responsible for the red, blue and purple colour of flowers, vegetables and fruits of higher plants. Anthocyanidins (e.g., peonidin, petunidin, pelargonidin, malvidin, cyanidin, and delphinidin) are mainly in glycosidic forms, which are commonly referred to as anthocyanins (Figure 3F).



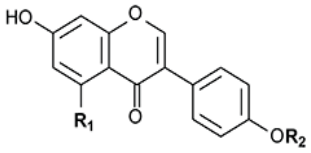
	R_1	R_2
Isorhamnetin	OCH_3	H
Kaempferol	H	H
Myricetin	OH	OH
Quercetin	OH	H



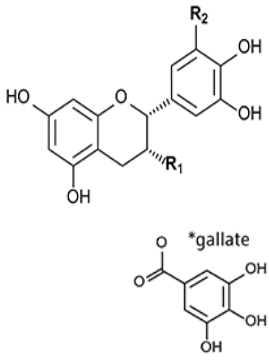
	R_1	R_2	R_3
Apigenin	H	OH	H
Luteolin	H	OH	OH
Baicalein	OH	H	H
Chrysin	H	H	H



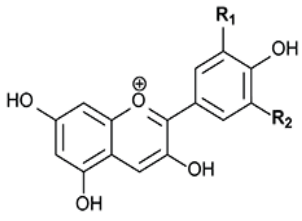
	R_1	R_2
Eriodictyol	OH	OH
Hesperetin	OH	OCH_3
Naringenin	H	OH



	R_1	R_2
Daidzein	H	H
Genistein	OH	H
Glycitein	OCH_3	H
Biochanin A	OH	CH_3
Formononetin	H	CH_3



	R_1	R_2
(-)-epicatechin	OH	H
(-)-epigallocatechin	OH	OH
(-)-epicatechin gallate	*gallate	H
(-)-epigallocatechin gallate	*gallate	OH



	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

Figure 3. Chemical structure of flavonoid subclass: structure of flavonols (A), flavones (B), flavanones (C), isoflavones (D), flavanols (E) and anthocyanins (F).

Polyphenolic amides

This group of phenolic compounds has N-containing functional substituents. Two relevant polyphenolic amides are capsaicinoids in chili peppers and avenanthramides in oats (Tsao, 2010). Capsaicinoids such as capsaicin are responsible for the hotness of chili peppers but have also been found to have strong antioxidant and anti-inflammatory properties, modulating the oxidative defence system in cells. Avenanthramides have also shown antioxidant activity including inhibition of LDL oxidation (Tsao, 2010).

Other polyphenols

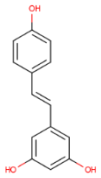
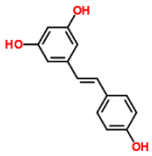
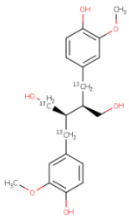
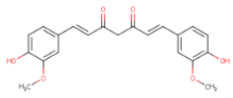
In addition to the phenolic acids, flavonoids and phenolic amides, there are other polyphenols in foods important to human health. Among these are stilbenes, and in this subgroup is resveratrol (Table 3), unique to grapes and red wine, *trans*-resveratrol, 3-*O*-glucoside, astringin, etc. Resveratrol's anticarcinogenic effects have been extensively studied, although it is found in such small quantities in the diet that any protective effect of this molecule would be unlikely at normal nutritional intakes (Manach et al., 2004).

Other type of polyphenols are lignans such as pinoresinol and derivatives (Table 3), which are present in oleaginous seeds (linseed), algae, leguminous plants (lentils), cereals (triticale and wheat), vegetables (garlic, asparagus, carrots), and fruit (pears, prunes).

Some dimeric polyphenols like curcumin (diferuloylmethane) in turmeric (Table 3) or rosmarinic acid, a dimer of caffeic acid found in rosemary, are important antioxidants in herbs and spices.

High molecular weight polyphenols are mainly hydrolysable tannins (ellagitannins and gallotannins), which are esters of gallic acid and ellagic acid with polyols, mainly glucose (Tsao, 2010), and condensed tannins, also known as procyanidins or proanthocyanidins, consisting of flavan-3-ol and flavan-3,4-diol units linked in polymers with a degree of polymerization as high as 50 and greater (Bravo, 1998).

Table 3. Other polyphenols in foods

Other typical polyphenols	Chemical structures
Resveratrol	
Trans-resveratrol	
Secoisolariciresinol	
Curcumin	

Consumption of polyphenols

Polyphenols are consumed daily throughout the world, although the literature about human polyphenol intake is still scarce. In 1998, the average dietary consumption of polyphenols was estimated at 1 g/day (Bravo, 1998), this figure is slightly above that reported in a more recent study carried out in the Spanish population 0.8 g/day (Tresserra-Rimbau et al., 2013). Kuhnau (1976) estimated the average daily intake of dietary flavonoids in the United States to be between 1 and 1.1 g/day, depending on the season, mainly consisting of flavonols, flavones, and flavanones. A more recent estimate of the dietary flavonoid intake by U.S. adults from US Department of Agriculture (USDA) is

189.7 mg/d, mainly from flavan-3-ols (83.5%), in the following foods: tea (157 mg), citrus fruit juices (8 mg), wine (4 mg), and citrus fruits (3 mg) (Chun et al., 2007). Comprehensive databases of the polyphenol content in foods such as the United States Department of Agriculture (USDA) Database for the Flavonoid Content of Selected Foods and the Phenol-Explorer databases by the French National Institutes for Agricultural Research have been developed for estimating polyphenol intake (Neveu et al., 2010; Rothwell et al., 2012).

In European adults, total flavonoid intake was 428 ± 49 mg/d, of which 136 ± 14 mg/d were monomeric compounds (Vogiatzoglou et al., 2015). According to Treserra-Rimbau et al. (2013), of the mean total polyphenol intake (820 ± 323 mg/day), 54 and 37% correspond to flavonoids and phenolic acids, respectively. Among these, hydroxycinnamic acids was the phenolic group most widely consumed and 5-caffeoylquinic acid was the most abundantly ingested individual phenolic compound. In Mediterranean countries, the main dietary source of phenolic compounds is coffee and fruits, olives and olive oil. The consumption of olives and olive oil was a differentiating factor in the phenolic profile of the Spanish population compared with other countries (Tresserra-Rimbau et al., 2013) (Table 4).

Regarding the French adult population, dietary intake of 337 polyphenols was investigated using Phenol-Explorer database. Mean total polyphenol intake was estimated at 1193 ± 510 mg/d (or 820 ± 335 mg/d when expressed as aglycone equivalents), with hydroxycinnamic acid esters and proanthocyanidins, being the most largely consumed. Non-alcoholic beverages and fruit were the most important contributors to polyphenol intake (Perez-Jimenez et al., 2011).

In healthy elderly Japanese people, total polyphenol intake was estimated at 1492 ± 665 mg/day, the greatest part provided by beverages (79.1%) and largely differing among individuals (183-4854 mg/day), also attributable mostly to beverage consumption. Coffee (43.2%) and green tea (26.6%) were the major sources of total polyphenols (Taguchi et al., 2015). In China (Li et al., 2013), the daily intake of total flavonoids, anthocyanidins, flavonols, flavones, isoflavones, and stilbene were 165.6, 27.6, 123.7, 10.7, 3.7 and 0.3 mg/day, respectively.

Table 4. Food sources and amount of total polyphenols, mostly eaten by the Spanish population.

Polyphenol type	Total Polyphenol (mg/d)	Food Sources
Hydroxycinnamic acids	276 ± 146	Coffee
Flavanones	132 ± 125	Oranges
Proanthocyanidins	117 ± 81	Red wine, apples
Flavonols	80.4 ± 32.7	Spinach, beans
Flavones	41.6 ± 26.1	Oranges
Anthocyanins	38.5 ± 37.4	Cherries, red wine
Catechins	26.7 ± 19.6	Apples, red wine

Data represents mean ± SD (adapted from Tresserra-Rimbau et al., 2013).

However, phenolic compound intake data is underestimated since the quantification of polyphenols ingested does not consider those associated with other food components such as dietary fiber, protein or phenolic compounds of high molecular weight not extracted with traditional methods (Bravo, 1998).

Attending to beneficial health effects associated to polyphenols, high recommended intake could be expected, although the dose-response of phytochemical effects is not linear and an excessive intake could lead to adverse effects (Holst and Williamson, 2008). However, the bioavailability of phenolic compounds is, in general, limited, as noted in the following section.

2.1.2. Bioavailability and metabolism of phenolic compounds.

The term “bioavailability” was originally used in pharmacology to define the concept of the “rate and extent to which a drug reaches its site of action” (D'Archivio et al., 2010). Applied to phenolic compounds, it is not only important to know how much of the compound is present in a specific food and its dietary intake, it is even more important to know how much of it is bioavailable and metabolised. Although a phenolic compound had a strong antioxidant or other biological activities, or bioactivities, *in vitro*, it could have little biological activity *in vivo* if little or none of the compound gets to the target tissues. The bioactivity of each polyphenol depends on the level of its activity and the extent to which it is absorbed, distributed, metabolized and eliminated from the body. In general, the

bioavailability of phenolic compounds depends on factors associated with food processing, with the host organism (sex, age, composition of intestinal microflora), as well as on interactions between polyphenols and other molecules (such as salivary proteins and digestive enzymes) (D'Archivio et al., 2010). Factors potentially affecting bioavailability, directly or decreasing polyphenol content in foods are illustrated in Table 5.

Table 5 Main factors affecting the bioavailability of dietary polyphenols in humans (D'Archivio et al., 2010).

External factors	**Environmental factors affecting phenolic content of foods (i.e., sun exposure, degree of ripeness); food availability**
Food processing related factors	Thermal treatments; homogenization; lyophilisation; cooking and methods of culinary preparation; storage
Food related factors	Food matrix; presence of positive or negative effectors of absorption (i.e., fat, dietary fibre)
Interaction with other compounds	Bonds with proteins (i.e., albumin, plant cell wall components) or with polyphenols with similar mechanism of absorption
Polyphenols related factors	Chemical structure; concentration in food; amount introduced
Host related factors	Intestinal factors (i.e., enzyme activity; intestinal transit time; colonic microflora). Systemic factors (i.e., gender and age; disorders and/or pathologies; genetics; physiological condition)

In general, after consumption polyphenols reach the small intestine and depending on their conjugation with sugars and solubility properties, are metabolised or absorbed during the digestion process. In the small intestine, they are mainly absorbed as aglycones or as simple glucosides and the remaining non-absorbed compounds (olygomers or polymers, rhamnosylglucosides, neohesperidosides, or esters with different compounds) reach the large intestine and are metabolised by the colonic microflora to render first aglycones and then simple phenolic acids (Manach et al., 2005; Lewandowska et al., 2013).

In the stomach, polyphenols are exposed to strong acidic conditions, which might influence their stability. However, *in vitro* digestion experiments carried out with different phenols, such as anthocyanins (Perez-Vicente et al., 2002), quercetin and caffeic acid derivatives (Bermudez-Soto et al., 2007), or olive oil phenols (Pereira-Caro et al., 2010), revealed that they were stable at low pH. In general, due to the acidic environment in the stomach and small gastric absorption area, phenolic compounds are poorly absorbed in the stomach and reach the small intestine (Manach et al., 2004). Exceptionally, few phenolic compounds, such as quercetin, may be absorbed in the stomach, as observed using a rat model where 38% of quercetin was rapidly absorbed in the stomach after *in situ* gastric administration (15 µmol/L) for 30 min, while rutin and isoquercitrin (quercetin-3-*O*-glucoside) were not hydrolysed nor absorbed (Crespy et al., 2002). Certain studies indicate that glucoside forms of phenolic derivatives could be absorbed in the stomach, such as malvidin-3-glucoside, which appeared in the plasma of rats after 6 min of anthocyanin administration (Passamonti et al., 2003) pointing to the presence of transporters in the stomach wall.

Physicochemical properties of polyphenols, including molecular weight, extent of glycosylation and esterification or structure are major determinants of intestinal absorption (Scalbert et al., 2002). Most flavonoids, except catechins and proanthocyanidins, are glycosylated in foods, which influences absorption through the gut barrier. Phenolic metabolites absorbed at the small or large intestine can be conjugated at the intestine cells to produce glucuronide, sulphate or methyl conjugates, and are transported by the portal vein to the liver. Phase II enzymes in this organ additionally conjugate polyphenols by glucuronidation, sulphation and/or methylation. This is part of the metabolic detoxification process common to many xenobiotics, which restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity (Manach et al., 2004).

Most polyphenols are poorly absorbed in the upper intestinal tract and reach the colon where they are extensively metabolised by the microflora into a wide array of low molecular weight phenolic acids (Figure 4). Gut bacteria can hydrolyse glycosides, glucuronides, sulphates, amides, esters and lactones. They also carry out reduction, ring-cleavage, decarboxylation, demethylation and dehydroxylation reactions. The hydrolysis of glycosides and glucuronides

typically results in metabolites that are potentially more biologically active than the parent compounds. In contrast, further bacterial transformation of aglycones can lead to more or less active compounds, depending on the substrate being metabolised and the products formed (Lampe and Chang, 2007). Differences in the bacterial strains that build up the colonic microbiota contribute to justify the large inter-individual variability in phenolic effects.

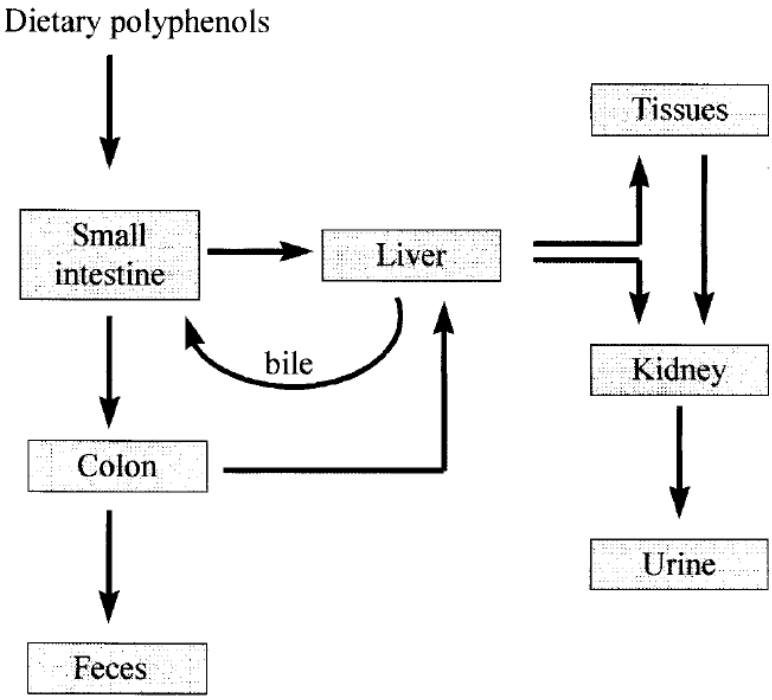


Figure 4. Possible routes of absorption for the consumed polyphenols in humans (from Scalbert and Williamson, 2000)

Phenols are extensively modified during the absorption: the glycosides could be hydrolysed in the small intestine or in the colon, and the released aglycones could be absorbed. Prior to the passage into the blood stream, phenols undergo other structural modifications due to the conjugation process, mainly in the liver. Plasma phenolic concentrations vary highly depending on the nature of the polyphenol and the food source. For instance, plasma concentrations of anthocyanins are very low, ranging from 10 to 50 nmol/L after a 50 mg of aglycone equivalent supply (Galvano et al., 2007).

Polyphenols and their metabolites are eliminated mainly in urine and bile. Polyphenols which are secreted in the bile suffer the enterohepatic recycling. They are secreted via the biliary tract into the duodenum where they are subjected to the action of bacterial enzymes (glucuronidases and sulphatases) in the small intestine and colon after which they may be reabsorbed (Manach et al., 2003), contributing to sustained plasma levels of phenolic metabolites (Figure 2).

2.1.3. Physiological properties and effects on health.

In the past years, research on dietary phenols has received great attention due to their role in human health. Phenolic compounds, as the most abundant antioxidants in our diets, may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases (CVD), inflammation and others (Scalbert and Williamson, 2000). Epidemiological studies have repeatedly shown an inverse association between the risk of chronic human diseases and the consumption of polyphenolic rich diets (Arts and Hollman, 2005; Pandey and Rizvi, 2009).

Polyphenol and cardiovascular diseases

Cardiovascular disease (CVD), including atherosclerosis, myocardial infarction, heart attacks, stroke, and cerebrovascular diseases, are a major healthcare problem and among the most costly diseases across many countries (Tarride et al., 2009). There are many factors involved in the aetiology and progression of CVD, such as hypercholesterolemia; due to high levels of low-density lipoprotein-cholesterol (LDL-C) or reduction of high-density lipoprotein-cholesterol (HDL-C), which are major risk factors for primary and established CVD. Elevated blood lipids have a close relationship with atherosclerosis, cardiovascular and cerebrovascular diseases. The serum total cholesterol (TC), triglyceride (TG), HDL-C, LDL-C, liver cholesterol and liver triglyceride levels are a reflection of the main indicators of lipid metabolism.

Numerous epidemiological and human intervention studies have suggested that regular consumption of polyphenol-rich foods, such as fruits, vegetables, chocolate/cocoa, tea and wine, may exert cardio-protective effects in humans (Hertog et al., 1997; Yochum et al., 1999; Nakachi et al., 2000; Sarriá et al., 2015). Prospective studies have indicated a correlation between the intake of

flavonols, flavones and flavanols and reduced risk of coronary artery disease (Arts and Hollman, 2005) as well as anthocyanin and flavanone intake and reduced CVD related mortality (Mariappan et al., 2007). Furthermore, meta-analyses have indicated that the consumption of three cups of tea per day reduces CVD risk by 11% (Peters et al., 2001), similarly regular, moderate red wine consumption is associated with a 32% reduced risk of CVD (Pai et al., 2006). These benefits may be attributed to polyphenols' capacity to inhibit LDL oxidation, which is a key mechanism in the development of atherosclerosis (Garcia-Lafuente et al., 2009). LDL oxidation is closely implicated in the development of atherosclerotic CVD, and both the concentration of oxidized LDL (ox-LDL) and the rate of LDL oxidation play an important role in CVD pathogenesis. Reducing LDL levels and improving LDL/HDL cholesterol ratios, notably by diet, are fundamental goals of CVD risk management (Habauzit and Morand, 2012).

Polyphenols and obesity and type 2 diabetes

Prevalence of obesity and type 2 diabetes mellitus (T2DM) is alarmingly increasing worldwide due to sedentary lifestyles, high fat diet regimens and genetic predisposition. Over the last 20 years, basic and translational studies have unraveled a strong biological relation between high glucose levels, impaired insulin signaling and vascular disease in the setting of T2DM which consists simultaneously with a cluster of conditions including low-grade inflammation, impaired insulin pathway and hyperglycemia. Potential biomarkers are protein kinase C (PKC), reactive oxygen species (ROS), advanced glycation end products (AGEs). interleukins, microRNA or myeloid calcifying cells (Paneni et al., 2014), among others. A number of related original publications have suggested that polyphenol-rich diets have the ability to protect against diabetes. It appears that anthocyanins or anthocyanin-rich foods intake is related to the risk of T2DM (Wedick et al., 2012; Anhê et al., 2013; Xiao and Hogger, 2015), with flavanols also playing a potential role (Martin et al., 2016).

In Zucker diabetic fatty rats, the ingestion of a cocoa-rich diet (10%), rich in flavanols, for 9 weeks attenuated hyperglycemia, improved insulin sensitivity, and increased β-cell mass and function. At molecular level, cocoa intake prevented β-cell apoptosis by increasing antiapoptotic proteins and decreasing proapoptotic proteins (Bax and caspase-3 activity). Moreover, in the pancreatic

tissue cocoa diet enhanced antioxidant defences (GPx and GR activities) to prevent the oxidative injury, i.e. lipid and protein oxidative damage was reduced (Fernandez-Millan et al., 2015). Cocoa and its polyphenolic compounds, mainly flavanols, might contribute to prevent or delay T2DM by modulating insulin secretion in β-pancreatic cells and targeting insulin-sensitive tissues because of their insulin-like activity or through the regulation of key proteins of the insulin signaling route. Among other actions, cocoa flavanols have been proved to enhance glucose uptake through the promotion of glucose transport, to repress glucose production, or to improve lipid metabolism (Martin et al., 2016).

Other researchers examined the association of dietary flavonol and flavone intake with T2DM and biomarkers of insulin resistance and systemic inflammation. None of total flavonols and flavones, quercetin, kaempferol, myricetin, apigenin, and luteolin was significantly associated with risk of T2DM (Song et al., 2005).

Some polyphenols such as quercetin, catechins, anthocyanidins, resveratrol and curcumin have been shown to modulate physiological and molecular pathways that are involved in energy metabolism, adiposity, and obesity (Meydani and Hasan, 2010: Hossain et al., 2016). The potential *in vivo* beneficial effects of these polyphenols on adiposity and obesity as complementary agents in the up-regulation of energy expenditure has emerged by investigating these compounds in cell cultures, animal models of obesity and in some human clinical and epidemiological studies (Meydani and Hasan, 2010).

In cell culture studies, resveratrol enhances lipolytic activity in adipocytes through induction of cAMP and inhibits adipogenesis in isolated human adipocytes (Szkudelska et al., 2009). Curcumin (5-20 μmol/L) suppressed 3T3-L1 differentiation, caused apoptosis, and inhibited adipokine-induced angiogenesis of human umbilical vein endothelial cells (Ejaz et al., 2009). In addition, in animal models, supplementing the high-fat diet of C57/BL mice with curcumin did not affect food intake but reduced body weight gain, adiposity, and microvessel density in adipose tissue (Ejaz et al., 2009). Green tea extract protects against nonalcoholic fatty liver disease by limiting hepatic lipid accumulation and injury without affecting hepatic antioxidant status and adiponectin-mediated lipid metabolism (Bruno et al., 2008). The most abundant green tea catechin, epigallocatechin gallate (EGCG), was studied for its effect minimizing diet-

induced obesity by increasing fat oxidation and decreasing leptin levels and energy absorption (Wolfram et al., 2005). Supplementation of the high-fat diet in a mice experiment with anthocyanins (cyaniding-3-glucoside) repressed body weight increase, decreased white and brown adipose tissue weights, and enhanced hyperinsulinemia by controlling the expression of enzymes involved in fatty acid and triacylglycerol synthesis, normalizing the mRNA level of TNF-α in the visceral adipose tissue (Tsuda et al., 2004).

Polyphenols and cancer

It is widely accepted that a high daily intake of fruits and vegetables helps to prevent the progression of cancer. Epidemiological studies indicate that there is an inverse correlation between regular fruit and vegetable consumption and the incidence of various types of cancer (Block et al., 1992), specifically stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas, and colon (Steinmetz and Potter, 1996). More recently, data from large cohort investigations have confirmed the inverse association (Franceschi et al., 1998; Benetou et al., 2008). However, there is certain controversy; some case-control studies described a scarce or null association between vegetable and fruit consumption and the incidence of bladder, pancreatic and stomach cancer (Botterweck et al., 1998; Larsson et al., 2006; Larsson et al., 2008). In the association between polyphenols and cancer risk, factors such as host genetic susceptibility, epigenetic modification, and gut microbiome patterns impact the role of polyphenols and may be responsible for the differences observed among studies (Wang et al., 2015). Dietary polyphenols can interfere at the initiation, development and progression of cancer through the modulation of different cellular processes, showing certain common signalling events, i.e. arrest of cell cycle by increasing levels of cyclin-dependent kinases (CDKs) and inhibition of cyclins, induction of apoptosis through cytochrome C release from mitochondria, activation of caspases and down- or up-regulation of Bcl-2 family members, inhibition of survival/proliferation signals (AKT, MAPK, NF-κB, etc.) and inflammation (COX-2, TNF-α, etc.), as well as suppression of key proteins involved in angiogenesis and metastasis (Ramos, 2008).

In vitro studies in cancer cell lines yield a valuable initial estimate of the effect of different agents on cell growth, proliferation, and apoptosis. For example, caffeic acid phenethyl ester (CAPE) suppressed the proliferation of

human melanoma cell line HO-1 and human glioblastoma cell line GBM-18 in a dose dependent fashion from 1.0 to 100 µg/mL. Major green tea polyphenols (epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate) were found to suppress growth of pancreatic cancer cells (Mia Pa Ca-2), lung tumor cells (H661 and H1299), colorectal carcinoma cells (HCT-116), head and neck carcinoma cells (H891), breast cancer (MCF-7) and endothelial and vascular smooth muscle cells (Nichenametla et al., 2006).

The mechanisms responsible for these anticancer effects of polyphenols have also been widely investigated. Carcinogens are known to modulate transcription factors (e.g., NF-κB, AP-1, STAT3), anti-apoptotic proteins (e.g., Akt, Bcl-2, Bcl-XL), proapoptotic proteins (e.g., caspases, PARP), protein kinases (e.g., IKK, EGFR, HER2, JNK, MAPK), cell cycle proteins (e.g., cyclins, cyclin-dependent kinases), cell adhesion molecules, cyclooxygenase 2 (COX-2), and growth factor signalling pathways (Aggarwal and Shishodia, 2006). Polyphenols have the potential to prevent and fight cancer through these multi-targeted approach: reducing DNA damage, inhibiting growth factor receptors, promoting the excretion of carcinogenic compounds, inducing apoptosis in cancer cells, inhibiting tumour cell invasion into surrounding tissues, inhibiting the formation of tumour blood vessels, decreasing inflammation by reducing free radical stimulation/activation of inflammatory pathways, etc. (Kampa et al., 2007; Pandey and Rizvi, 2009). For instance, an olive oil polyphenolic extract has shown strong inhibitory effects on the growth of colon adenocarcinoma cells through the inhibition of p38/CREB signalling, a decrease in COX-2 expression and the stimulation of a G2/M phase cell cycle block (Corona et al., 2007). The anti-cancer activity of green tea extracts on human cervical adenocarcinoma HeLa cells has been attributed to the pro-oxidant and anti-proliferative activities of tea polyphenols (Krstic et al., 2015).

Ellagic acid has been shown to inhibit chemically induced cancer in the lung, liver, skin and oesophagus of rodents, as well as TPA-induced tumour promotion in mouse skin (Stoner and Mukhtar, 1995). More examples of the effects of bioactive polyphenolic compounds commonly found in many fruits and vegetables in *in vivo* studies on cancer are found in the comprehensive reviews by Stoner and Mukhtar, 1995, and Nichenametla et al., 2006.

2.2. Beverages and plant by-products as a source of dietary polyphenols.

In response to the general concern of consumers on their health, in recent years there has been an evolution from a healthy and balanced diet towards a health-promoting diet, searching foods that promote health and well-being. This has led to changes in the dietary habits, with the introduction of foods and beverages not previously consumed in our country (e.g. green tea or yerba mate) and an increased intake of dietary supplements and functional foods (i.e. foods supplemented with ingredients with proved health beneficial effects).

2.2.1. Grape, wine and winery by-products.

Grape (*Vitis vinifera* L.) is one of the most abundant fruit crops and also among the richest in polyphenolic compounds. Grape, wine and winery by-products have great economic value. Italy, France, Spain, and the United States are among the most important producers in the World. In 2013, 69 million tons of grapes were produced, with almost 29 million tons corresponding to European contributors (FAOSTAT-FAO Statistical Database, 2016). The winemaking process is based on ancestral procedures. Approximately 80% of grapes are used in winemaking and ~20% of the weight of processed grapes remains as pomace (Kammerer et al., 2004). For example in Europe, it is estimated that 3.2 million tons of grape by-products including skins, seeds and stems, were produced in 2013 (FAOSTAT-FAO Statistical Database, 2016). The major residues from wine-making activity are organic wastes (grape pomace, containing seeds, pulp and skins, grape stems, and grape leaves), and wastewater, together with emission of greenhouse gases (CO_2, volatile organic compounds, etc.), and inorganic wastes (diatomaceous earth, bentonite clay, and perlite) (Teixeira et al., 2014). Grape pomace (GP) is the winery waste originated during the production of must (grape juice) by pressing whole grapes. The use of winery by-products is becoming a hot topic. So far, they are employed in producing juices, jam, yogurts, jelly, colorants, as a source of dietary fibre and functional ingredients (Mildner-Szkudlarz et al., 2011; Chouchouli et al., 2013). In the last decade, the interest in grape by-products has increased due to the potential health benefits and these products which have been commercialised as nutraceuticals or dietary supplements (Torres and Bobet, 2001; Georgiev et al., 2014).

2.2.1.1. Phenolic compounds in grape by-products.

As mentioned above, GP mainly consist of the skins, seeds and stems. Due to poor extraction during the production of wine, GP contains a high amount of phenolic compounds, up to 10-11% of the GP dry weight (Makris et al., 2007). The phenolic content of grape depends on factors associated with the varieties of grape, climate, cultivars, irrigation, management practices, degree of ripeness, berry size, food processing and grapevine variety (Jackson and Lombard, 1993; Rodríguez-Montealegre et al., 2006; Ivanova et al., 2011).The phenolic composition of GP is variety dependant; regardless, anthocyanins, catechins, procyanidins, flavonol glycosides, phenolic acids and stilbenes are the principal phenolic constituents in GP (Yu and Ahmedna, 2013).

Red varieties are richer in anthocyanins than white varieties, which mostly lack this type of flavonoids. Cantos et al. (2002) analysed the polyphenol composition of four red and three white table grape varieties by HPLC-DAD-MS and found that anthocyanins were the main phenolics in red grapes ranging from 69 (Crimson Seedless) to 151 (Flame Seedless) mg/kg fresh weight of grapes, whereas flavan-3-ols, specifically gallocatechin, procyanidin B1, procyanidin B2, procyanidin B4, procyanidin C1, catechin and epigallocatechin, were the most abundant phenolics in the white varieties ranging from 52 (Dominga) to 81 (Moscatel Italica) mg/kg fresh weight of grapes.

Regarding grape by-products, there is a greater concentration of phenolic compounds in the seeds than in the skin for almost all the studied varieties (Obreque-Slier et al., 2010; Ćurko et al., 2014; Di Lecce et al., 2014). Grape skin proved to be a rich source of anthocyanins (in red varieties), hydroxycinnamic acids, flavanols, flavonol glycosides, procyanidins and stilbenes, whereas flavanols were the most abundant in seeds (Yu and Ahmedna, 2013). A study on the phenolic composition of skin and seeds of ten grapes *Vitis vinifera* varieties grown in a warm climate showed that grape skin contained tartaric esters of hydroxycinnamic acids (6–45 mg/kg of grape), monomeric and dimeric flavan-3-ols (9–96 mg/kg) and flavonols (25–197 mg/kg), whereas the seed constituents comprised almost exclusively flavan-3-ols and procyanidins with a concentration ranging between 330–1390 mg/kg (Rodríguez-Montealegre et al., 2006).

Proanthocyanidins (condensed tannins) and anthocyanins are two important polyphenolic constituents of red grapes that are partly extracted during

wine-making and contribute to the chemical and sensory properties of wine, including colour, bitterness, and astringency. Proanthocyanidins are located in the seed and skin of the grape. Seed proanthocyanidins contain a higher proportion of galloylated procyanidins, whereas those from skin contain prodelphinidins (Hernandez-Jimenez et al., 2009). Although the phenolic composition can vary strongly depending on the variety and cultivation conditions, the skin contains the highest amounts of tannins in the grape berry. A study on the composition of grape (*Vitis vinifera* L. cv. Shiraz) skin proanthocyanidins showed that there were variations depending on the berry development stage, so that the development was correlated with an increase in proanthocyanidin mean degree of polymerization, the proportion of (-)-epigallocatechin extension subunits, and the content of anthocyanins (Kennedy et al., 2001).

Resveratrol is the main stilbene found in grape skins, mostly as *trans*-resveratrol, although methylated and glucosilated forms (pterostilbene and piceid, respectively) and oligomeric structures (vitiferins) have also been described (Waterhouse, 2002).

With respect to grape seeds, the relative proportion of seeds ranges from 38 to 52% of the dry material. Polyphenols in grape seeds are essentially flavonoids, particularly flavanols (catechin, epicatechin and epicatechin-3-*O*-gallate monomers) and polymers (Yu and Ahmedna, 2013). Table 6 shows the levels of gallic acid, catechin, and epicatechin in seeds or skins of Muscadine, Chardonnay, and Merlot grapes, with lower concentrations in winery by-product grape skin than in seeds (Yilmaz and Toledo, 2004).

Table 6. the phenolic content in different grape varieties.

Grape varieties	**Phenolic content (mg/100 g of dm)**		
Grape sample	**Gallic acid**	**Catehin**	**Epicatechin**
Seed			
Chardonnay	15	358	421
Merlot	10	127	115
Skin			
Chardonnay	5	60	44
Merlot	3	16	13

d.m.: dry matter

Results on the composition of seed phenolic extracts from grape varieties grown around the world were similar, comparing two cultivars grown in Croatia (Ćurko et al., 2014) and in Brazil (Pinot Noir, Isabel, Sangiovese, Negro Amaro, Cabernet Sauvignon, and Primitivo) (Rockenbach et al., 2011). The grape seed extracts of both origins were rich sources of proanthocyanidins, usually oligomers and polymers of polyhydroxy flavan-3-ols such as (+)-catechin and (−)-epicatechin, mainly in the form of gallate esters.

2.2.1.2. Health beneficial effects of grape by-products.

Cardiovascular diseases

Recent observational and clinical studies suggest that the consumption of wine and grape products, among other foods rich in polyphenols, is associated with decreased risk of CVD (Dohadwala and Vita, 2009). CVD is associated with modifications in fatty acid metabolism and excessive lipid oxidation of LDL. Several studies have shown that consumption of grape products may have beneficial effect on cardiovascular system by reducing oxidative stress, improving endothelial and vascular function, inhibiting platelet aggregation, decreasing LDL oxidation, altering blood lipids, and modulating the inflammatory process (Dohadwala and Vita, 2009; Wightman and Heuberger, 2015).

Grape phenolic extracts possess many biological activities associated with cardioprotection, which have shown to regulate plasma lipid levels and inhibit oxidation of human low-density lipoproteins (Xia et al., 2010). *In vitro* studies have demonstrated that grape-derived flavonoids and resveratrol limit *ex vivo* LDL oxidation (Frankel et al., 1993). Red grape juice polyphenols alter cholesterol homeostasis and increase LDL-receptor activity in human cells *in vitro* (Davalos et al., 2006). The effect of consuming red wine or its major polyphenol constituents, catechin or quercetin, on the development of atherosclerotic lesions, in relation to the susceptibility of plasma LDL to oxidation and to aggregation, was studied in atherosclerotic apolipoprotein E deficient (E°) mice. The study showed that the hypercholesterolemic mice that consumed wine polyphenols for 6 weeks had markedly less atherosclerosis than control animals, which was associated with protection against LDL oxidation (Hayek et al., 1997).

There are epidemiological evidences on the cardiovascular benefits of grape consumption, including the classical study by Renaud and de Lorgeril (1992) that led to the term “French Paradox”, showing a lower mortality from coronary heart disease in the French population in spite of its high saturated fat intake, which was attributed to the high consumption of red wine. In addition, many clinical studies support the beneficial effects of grape polyphenols. Among these studies, a 2 week intervention in coronary artery disease patients consuming a purple grape juice, improved flow-mediated vasodilation and reduced LDL susceptibility to oxidation, being improved endothelium-dependent vasodilation and prevention of LDL oxidation the potential mechanisms by which cardiovascular events were prevented, independent of alcohol content (Stein et al., 1999). In accordance, in healthy men and women moderate red wine consumption for 4 weeks was associated with a significant 11–16% increase in fasting HDL-cholesterol and 8–15% decrease in fasting fibrinogen compared with drinking water with or without red grape extract (Hansen et al., 2005).

Obesity and type 2 diabetes

Polyphenols in grapes and grape products may reduce metabolic syndrome and prevent the development of obesity and type 2 diabetes acting as multi-target modulators with antioxidant and anti-inflammatory effects (Chuang and McIntosh, 2011). In rats fed a high-fat diet, grape-seed procyanidins reduced proinflammatory molecules: C-reactive protein (CRP), interleukin (IL)-6 and tumour necrosis factor (TNF)-α expression but enhanced the expression of the anti-inflammatory cytokine adiponectin (Terra et al., 2009). Similarly, in hamsters fed a high-fat diet for 12 weeks, Chardonnay grape seed polyphenolic extract reduced obesity development through the regulation of plasma glucose, triglycerides, insulin, and leptin levels as well as related metabolic pathways including adipokine secretion and oxidative stress (Décordé et al., 2009). Such effects have also been observed in obese, type 2 diabetes mellitus patients in a double-blinded randomized crossover trial, in which a grape seed extract (600 mg/day) or placebo was consumed for 4 weeks. Markers of inflammation and glycaemia significantly improved after consuming the extract (Kar et al., 2009).

Cancer prevention

Phenolic compounds in grapes and/or grape-related products have been associated to promising chemopreventive effects in both *in vitro* and preclinical

models. Some of the mechanisms of action involved have been characterized. Phenolic compounds in muscadine grapes showed anticancer properties by inhibiting cancer cell viability and inducing cell apoptosis in two colon cancer cell lines (HT-29 and Caco-2) (Yi et al., 2005). Grape skin polyphenols presented antitumor and antimetastatic activities in a murine model of breast cancer. Metastasis of tumour cells to the lungs was inhibited by grape skin extracts (0.5 and 1.0 mg/ml in drinking water) and the survival of the mice was enhanced (Sun et al., 2012). In a UVB radiation-induced mouse skin carcinogenesis model, feeding grape skin polyphenols was effective in preventing UVB-induced oxidative stress and mitogen-activated protein kinases (MAPK) proteins as well as activating nuclear factor-κB (NF- κB) and restoring MAPK phosphatases in SKH-1 hairless mice (Akhtar et al., 2007). Anticancer effects of whole black grape (seeds included) extracts have also been reported in human cancerous colon tissue via inhibition in DNA turnover enzymes (Durak et al., 2005). Grape-seed procyanidins also inhibited the proliferation of pancreatic carcinoma cells by cell cycle blockage or apoptotic induction and invasiveness was also suppressed through down-regulation of matrix metalloproteinases MMP-2 or MMP-9 (Chung et al., 2012).

2.2.2. Coffee and yerba mate as a source of hydroxycinnamic acids.

Green coffee and yerba mate are two products characterized by their high content in antioxidant bioactive compounds (polyphenols, mainly hydroxycinnamic acids) and thus both present great health potential.

Yerba mate (*Ilex paraguariensis* St. Hilaire) is used for the preparation of the most popular tea-like beverage in many countries of South America, such as Argentina, Paraguay, Uruguay, and southern Brazil. Argentina is the largest producer, cultivating around 152.000 hectares per year in the northeast part of the country (states of Misiones and Corrientes). This is equal to approximately 300.000 tons per year, which is mainly consumed locally, representing a large portion of the country's gross domestic product. Brazil and Paraguay are the 2nd and 3rd largest producers, respectively. *Ilex paraguariensis* dried and minced leaves are made into a brewed tea, which has evolved from a tea drunk by the Guarani ethnic group to a beverage that has a social and almost ritualistic role in many South American modern societies (Grigioni et al., 2004; Heck and González de Mejia, 2007).

Coffee is one of the most popular beverages all over the world. The two main commercial coffee varieties of green coffee beans are *Coffea arabica* L. (Arabica) and *Coffea canephora* L. (Robusta), from the major growing regions of America, Africa, Asia, and Oceania (Alonso-Salces et al., 2009a). During coffee production, the green coffee beans are heated to 200-240°C for 10-15 min depending on the degree of roasting required which is generally evaluated by the weight loss of the sample thermally treated. The roasting process gives rise to a large number of volatile compounds that confer coffee its pleasant taste and aroma (Schenker et al., 2002). However, the roasting process also leads to profound changes in the chemical composition and biological activities of coffee, including the transformation of naturally occurring substances in green coffee and the generation of compounds derived from the Maillard reaction, carbohydrate caramelization, and pyrolysis of organic compounds (Daglia et al., 2000). Phenolic composition, concentration and antioxidant activity of the coffee beans is also influenced by roasting.

2.2.2.1. Phenolic compounds in coffee and mate.

Green coffee and mate have similar chemical composition, rich in phytochemicals. Polyphenols, as key bioactive compounds, are present in concentrations of 6-12% and 8-10% of dry matter in coffee (Trugo and Macrae, 1984; Chu et al., 2009) and mate (Chandra & Gonzalez de Mejia, 2004; Bravo et al., 2007), respectively. These concentrations represent approximately 10 g/L, therefore being beverages very rich in antioxidant compounds. Qualitatively, the phenolic composition in both products is similar, being mostly hydroxycinnamic acids: caffeoylquinic acid isomers [3-*O*-caffeoylquinic or neochlorogenic (3-CQA), 4-*O*-caffeoylquinic or criptochlorogenic (4-CQA), 5-*O*-caffeoylquinic or chlorogenic (5-CQA) acids], and dicaffeoylquinic acids [3,4-dicaffeoylquinic (3,4-diCQA); 3,5-dicaffeoylquinic (3,5-diCQA); 4,5-dicaffeoylquinic (4,5-diCQA)], together with feruloylquinic acids [3-feruloylquinic (3-FQA); 4-feruloylquinic (4-FQA) and 5-feruloylquinic (5-FQA)], collectively known as chlorogenic acids (CGAs). Also, in much lower concentrations, simple phenolic acids (caffeic and ferulic acids), amides and glycosides of cinnamic acids and other hydroxycinnamates have been described (Bravo et al., 2007; Alonso-Salces et al., 2009a).

The total polyphenol concentration of yerba mate, measured by the Folin–Ciocalteau method ranged from 90 to 176 mg gallic acid equivalents (GAE)/g dry leave in traditional mate tea (de Mejía et al., 2010). A commercial variety of mate showed a phenolic content of about 8 g/100g D.M. (Bravo et al., 2014), which is higher than that of green tea and similar to red wines. Mate naturally contains a wide range of polyphenols, methylxanthines, and caffeoyl derivatives. Yerba mate cinnamoylquinic acids (caffeic acid, chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid) account for over 90% of the total phenolic content. Caffeic acid reaches 2.1% of the phenolic composition. The rest are flavonoids (quercetin, rutin and kaempferol). Flavonols are present around 4.7 mg/g dry mate. Kaempherol accounts for 90% of the flavonols in mate leaves, which also contain the methylxanthines caffeine and theobromine (Bracesco et al., 2011; Bravo et al., 2014). 3,5-diCQA and 5-CQA are commercially available and have been extensively studied due to its antioxidant activity, which is similar to epigallocatechin gallate (EGCG) (Baeza et al., 2014).

Total polyphenols in green coffee range from 3.04% to 4.08% in defective coffee beans and 3.36% to 4.54% as GAE in graded beans (Ramalakshmi et al., 2007). The profiles of phenolic compounds and methylxanthines of the green beans of the coffee varieties Arabica and Robusta have been previously detected by liquid chromatography coupled with ultraviolet absorbance detection, electrospray ionization, collision-induced dissociation and tandem mass spectrometry on a triple quadrupole (Alonso-Salces, et al., 2009b). Three phenolic acids (caffeic acid, ferulic acid and dimethoxycinnamic acid), and many different isomeric caffeoylquinic, feruloylquinic, *p*-coumaroylquinic, dicaffeoylquinic, diferuloylquinic acids and other esters have been identified, showing the phenolic complexity of green coffee beans, together with three methylxanthines (caffeine, theobromine and theophylline) (Alonso-Salces, et al., 2009a). Mainly, the phenolic compounds of green coffee are chlorogenic acids, cinnamoyl amides, cinnamoyl glycosides, and free phenolic acids (Alonso-Salces et al., 2009b). CGAs and related compounds reach levels up to 14 % (dry matter basis), and may vary from 4 to 8.4% in *Coffea arabica* and from 7 to 14.4% in *Coffea canephora* (Farah and Donangelo, 2006).

During coffee processing, CGAs may be isomerized, hydrolysed or degraded into low molecular weight compounds accompanying Maillard reaction,

even though up to 60% of total CGAs in the green coffee beans can be lost with conventional roasting conditions (Trugo and Macrae, 1984).

Methylxanthines are another important group of bioactive compounds in green coffee and mate. Caffeine is the most abundant methylxanthine, together with theobromine and theophylline (Ito et al., 1997; Alonso-Salces et al., 2009a; Bravo et al., 2014). Caffeine consumption in a cup of mate is approximately 78 mg. Compared with coffee, the caffeine content per cup of mate is similar; however, the usual consumption of mate prepared in the traditional way as mate "cebado" can be as high as 0.5-1 L/day, with a daily intake of 260-520 mg of caffeine or higher (Mazzafera, 1997).

Terpenoids is another group of compounds determining the effects derived from consumption of these beverages. Two diterpens have been identified in coffee, caffestol and kahweol, responsible for its aroma, with concentrations of 0.6% dry matter (Kurt and Speer, 2002). Arabica coffee is richer in diterpens than Robusta. These compounds have hypercholesterolemic effects, being partly responsible for the negative effects of high coffee consumption (Bonita et al., 2007). Mate is rich in triterpenoids saponins, which are bitter compounds responsible for the organoleptic properties of mate. The main saponins identified in *Ilex paraguarienses* contains ursolic acid and are called matesaponins 1, 2, 3, 4 and 5. Concentrations of these compounds of up to 5.5 mg/g dry leaves (Baeza et al., submitted) and 352 µg/mL have been reported in beverages prepared from 15 g of dry leaves in 100 mL water (Gnoatto et al., 2005). Some beneficial properties of mate (anti-inflammatory, hypocholesterolemic) have been attributed to saponins (Ferreira et al., 1997; Gnoatto et al., 2005).

2.2.2.2. Health beneficial effects of coffee and yerba mate.

The many healthy properties attributed to mate have led to its use as a dietary supplement in Western countries. However, due to the immigration of South Americans, among other factors, it is nowadays more commonly consumed as a beverage, made by steeping the leaves of the plant in hot water. Nevertheless, it is necessary to better understand the health effects of this beverage as well as the mechanisms of action because although mate has been

related with health promoting effects, some of the existing data is contradictory, and its consumption has also been associated with an increased incidence of certain types of cancer.

In the last decades, the scientific evidence supporting the health benefits of yerba mate and green coffee has grown. Coffee is one of the main dietary sources of antioxidants due to its high phenolic content, although antioxidant activity has also been attributed to caffeine and its metabolites (Shi et al., 1991) or to melanoidins (Delgado-Andrade et al., 2005a; Delgado-Andrade et al., 2005b). Similarly, many *in vitro* and *ex vivo* studies have shown that mate is a potent antioxidant (Clifford and Ramirez-Martinez, 1990; Gugliucci and Stahl, 1995; Ricco et al., 1995; Gugliucci, 1996; Mazzafera, 1997; Filip et al., 2000; Schinella et al., 2000; Filip et al., 2001; Actis-Goretta et al., 2002; Gugliucci and Menini, 2002; Bracesco et al., 2003; Chandra and Gonzalez de Mejia, 2004; Bixby et al. 2005; Anesini et al., 2006; Bravo et al., 2007) with a strong reducing power, free radical scavenging activity or inhibiting human LDL oxidation, enzymatic and non-enzymatic lipid peroxidation in rat liver peroxisomes, peroxidation of red blood cell membranes or H_2O_2-induced breakdown of DNA double chain, etc. Strong antioxidant effects have also been observed in *in vivo* animal (Miranda et al., 2008; Martins et al., 2009; Bravo et al., 2014) and human studies (Gugliucci and Stahl, 1995; Ricco et al., 1995; Gugliucci, 1996; Schinella et al., 2000; Gugliucci and Menini, 2002; Bixby et al., 2005; Miranda et al., 2008; Martins et al., 2009; Matsumoto et al., 2009).

Many of the health beneficial effects of green coffee and mate have been linked to the antioxidant effect of their phenolic fraction. Therapeutic properties traditionally assigned to mate are due to its hypolipemic, hepatoprotector, choleretic, anti-obesity, diuretic, anti-inflammatory and anti-ageing effects (Gorzalczany et al., 2001; Pittler and Ernst, 2004; Heck and Gonzalez de Mejia, 2007; Gugliucci et al., 2009). Also, several studies suggest that moderate coffee consumption would reduce risk of type-2 diabetes, liver cirrhosis, colorectal or hepatic cancer, or inflammatory and CVD (Giovannucci et al., 1998; Ruhl and Everhart, 2005; Shimazu et al., 2005; Greenberg et al., 2006; Bonita et al. 2007; Nkondjock, 2009; Bravo et al., 2016). However, there is controversy related to the association of mate consumption with cancer. For instance, mate consumption has been associated with esophageal, oral, lung and bladder cancers (Loria et

al., 2009) and oropharyngeal cancer (Goldenberg, 2002), mostly attributed to the high temperature mate is traditionally consumed.

Cardiovascular disease

One of the most widely accepted health effects of mate is its cardioprotective potential associated to its hypocholesterolaemic, antithrombotic, antiatherogenic and hypotensor properties, improving endothelial function and vascular reactivity (Muccillo-Baisch et al., 1998; Filip et al., 2000; Paganini-Stein et al., 2005; Schinella et al., 2005; Andersen et al., 2006; Mosimann et al., 2006; De Morais et al., 2009; Schinella et al., 2009; Bravo et al., 2014). These effects have been described in *ex v*ivo (De Morais et al., 2009; Schinella et al., 2009) and *in vivo* in animals (Muccillo-Baisch et al., 1998; Mosimann et al., 2006; Martins et al., 2009; Bravo et al., 2014), as well as in human intervention studies in hyperlipidemic volunteers (De Morais et al., 2009), where a relevant contribution of mate drinking to the hypocholesterolaemic effect of statins was observed. Regular consumption of mate tea may increase antioxidant defenses by multiple mechanisms, such as lowering lipid peroxidation and increasing mRNA levels of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase (Matsumoto et al., 2009).

The effect of yerba mate on serum lipids and antioxidant status in hypercholesterolaemic rats has been studied; mate consumption had no effect on HDL-C or protein carbonyls, yet it showed a marked hypolipidaemic action, decreasing TG, total and LDL-cholesterol, and serum malondialdehyde (MDA) levels, as a marker of lipid oxidation, that had been increased after consuming the high-cholesterol diet (Bravo et al., 2014). Gao et al. (2013b) investigated the antihyperlipidemic and antioxidant effects of mate in a hyperlipidemic rat model and observed that total body weight was reduced and serum levels of LDL-C were lowered, whereas levels of HDL-C were increased. In addition, the activities of superoxide dismutase and glutathione peroxidase in serum were elevated, whereas the levels of MDA decreased. These results pointed to mate strongly reducing the risk of atherosclerosis. In addition, according to a study carried out by the same group, yerba mate reduced endothelin and thromboxane B2 levels and increased nitric oxide (NO) and 6-keto prostaglandin F1α blood levels and reduced the pathological damage of vascular endothelial cells, decreased intercellular adhesion molecule-1 (ICAM-1) protein expression, and up-regulated

mRNA expression of hepatic LDL receptor and scavenger receptor B1 (Gao et al., 2013a). Other authors investigated the vasorelaxing properties of the aqueous and n-butanolic extractable fractions from yerba mate leaves. Yerba mate induced vasodilatation in standard-diet rats in a dose-dependent manner and the hypercholesterolaemic diet substantially reduced the effect of mate. Yerba mate extract attenuated the myocardial dysfunction induced by ischemia and reperfusion; in the diminution of oxidative damage, a nitric oxide-dependent mechanism was involved (Schinella et al., 2005).

In hypercholesterolaemic subjects on long-term statin therapy, regular consumption of mate (330 mL, 3 times/day) promoted additional 10.0 and 13.1% reductions in LDL-C after 20 and 40 days, respectively, and increased HDL-cholesterol by 6.2% after 40 days (de Morais et al., 2009). In a randomized, double-blind, placebo-controlled trial, yerba mate tea or placebo (5 g/day) was administered to different groups for 6 weeks. In the yerba mate group, whole blood viscosity, plasma viscosity and the Equation K value of erythrocyte sedimentation rate (ESRK) decreased significantly (Yu et al., 2015). Yerba mate also had beneficial effects in individuals with high blood viscosity and microcirculatory disturbance (De Morais et al., 2009).

On the other hand, it has been suggested that coffee consumption can increase the risk of coronary heart disease, increasing blood pressure, plasma cholesterol, LDL oxidation, homocysteine and C-reactive protein levels. However, it is important the way in which coffee is consumed, not only whether it is decaffeinated coffee or not, considering the possible hypertensive effects of caffeine, but also if the coffee is filtered or non-filtered (boiled) due to the higher diterpene content in the later, with a well-known hypercholesterolemic effect in humans (Van Rooij et al., 1995; Bonita et al., 2007). In spite of this, moderate consumption of coffee seems to have a cardioprotective effect (Bonita et al., 2007; Mineharu et al., 2009) attributed to the phenolic content with a beneficial effect counteracting the deleterious effects of caffeine and diterpenes.

Although there are many studies on the effect of roasted coffee on CVD (reviewed in Bravo et al., 2016), evidences on the effect of green coffee are still scarce. The higher hydroxycinnamic acid content in green coffee could be associated with a potential improved effect on cardiovascular health compared to roasted coffee. A reduction on blood pressure and improved endothelial function

in hypertensive patients have been observed after consumption of chlorogenic acid or green coffee (Kozuma et al., 2005; Watanabe et al., 2006; Ochiai et al., 2009), with an improved vasoreactivity and decreases homocysteine levels (Ochiai et al., 2004). The improved endothelial function and blood pressure by 5-CQA in green coffee could be inhibited by hydroxyhydroquinone formed during coffee roasting (Yamaguchi et al., 2008; Ochiai et al., 2009), which would support the apparent higher cardioprotective effect derived from green coffee consumption.

A green coffee bean extract (GCBE) has shown to reduce hypertension in both spontaneously hypertensive rats and humans. Oral ingestion of the extract, as well as 5-CQA decreased blood pressure in spontaneously hypertensive rats, being ferulic acid (FA) as the metabolite of 5-CQA, the candidate hypotensive component (Suzuki et al., 2002). Effects of GCBE on blood vessels were evaluated in healthy males. The reactive hyperaemia ratio (RHR) in the test drink group containing GCBE (5-CQA: 140 mg/day), began to increase after ingestion for 1 month and was significantly higher than that in the placebo group after ingestion for 3 months and 4 months. In addition, after ingestion for 4 months, the test drink group showed a significant decrease in the plasma total homocysteine level compared with the pre-ingestion level (Ochiai et al., 2004). A randomized, double-blind, placebo-controlled, parallel group study evaluated the dose-response relationship of GCBE in male volunteers with mild hypertension. Results showed that blood pressure was significantly reduced in a dose-related manner (46 mg, 93 mg, or 185 mg of GCBE once a day; Kozuma et al., 2005). Caffeoylquinic acids are the compounds mainly responsible for the beneficial cardiovascular effects of mate and for counteracting the potential negative effects of coffee. 5-CQA and caffeic acid have shown to have hypotensive properties, increasing the bioavailability of nitric oxide, and decreasing the endothelial production of ROS, resulting in an improved endothelial function. Moreover, protective effect of 5-CQA on paraoxonase-1 in human lipoproteins has been described, thus preserving its protective role against LDL oxidation (Gugliucci and Bastos, 2009). Recently, it has been reported the inhibitory effects of 5-CQA on cyclooxigenases (COX) 1 and 2, and P-selectine expression in platelets (Park, 2009), blocking platelet activation. Also, one of the major metabolites of hydroxycinnamic acids, dihydrocaffeic acid, is able to scavenge ROS in endothelial cells increasing the nitric oxide synthase (NOS) activity, contributing

to the improved endothelial function mediated by endothelial NOS (eNOS) (Huang et al., 2004). Another cardioprotective and anti-atherosclerotic mechanism of dicaffeoylquinic acids in mate and coffee is through angiotensin II inhibition in vascular smooth muscle cells and the protection of endothelial cells against oxidative stres (Chen et al., 2007 and Chiou et al., 2009).

Obesity and type 2 diabetes

The hypolipemic effect of green coffee and mate has a clear beneficial role against obesity. In mice fed a high fat diet, yerba mate decreased the differentiation of preadipocytes and reduced accumulation of lipids in adipocytes, dropping the growth of adipose tissue and lowering body weight gain, thus at the end, decreasing obesity (Kang et al., 2012). Also in obese mice, a yerba mate extract showed marked attenuation of weight gain, adiposity, decreased epididymal fat-pad weight, and in addition, serum levels of TC, TG, LDL-C, and glucose were restored (Arcari et al., 2009). In accordance, in another study in obese animals, mate consumption significantly reduced body and visceral fat weights, adipocyte size, plasma and liver lipid concentrations, blood glucose, insulin and leptin. Moreover, mate counteracted the high-fat diet effect by means of regulating the expression of certain transcription factors, receptors and uncoupling proteins (SREBP1c, PPAR-γ2, UCP2, UCP3) and key enzymes in lipolysis and lipogenesis (CPT-1, MCD, FAS, HMGCR) in adipose tissue, thus showing the potent anti-obesity activity of mate and the molecular mechanisms involved (Pang et al., 2008). Another study supports that yerba mate extract may down-regulate the expression of genes that are involved in adipogenesis, such as Creb-1 and C/EBPα, and in contrast the extract up-regulated the expression of genes related to the inhibition of adipogenesis (Arcari et al., 2013). In humans, regular consumption of mate has also shown modulating effects on the expression of several genes related to obesity (Martinet et al., 1999; Oliveira et al., 2008a; Matsumoto et al., 2009). In fact, mate has been used as a dietary supplement in sliming strategies as mate may delay gastric emptying with the subsequent satiating effect (Andersen and Fogh, 2001).

Regarding the effect of green coffee on obesity, an animal study showed a reduction in body weight and visceral fat after consumption of a green coffee extract, caffeine or hydroxycinnamic acids, via reduced fat absorption and activation of hepatic lipid metabolism (Shimoda et al., 2006). Many animal,

human and epidemiological studies on roasted coffee (caffeinated, decaffeinated, instant coffee, etc.) show a reduction on body weight, fat accumulation, reduction of adipocyte number, etc. It is suggested that the effect of coffee decreasing body weight and adiposity is due to increased thermogenic, beta-oxidation and lipolysis, effects mainly associated to its caffeine content, which would also increase physical activity and energy expenditure (Greenberg et al., 2006). Similarly, coffee would have a satiating effect, decreasing serum leptin levels (Kovacs et al., 2004) and modulating the levels of gastric hormones involved in appetite regulation and insulin secretion (Johnston et al., 2003; Tunnicliffe and Shearer, 2008). Although these effects have been attributed to caffeine, the high CGAs content of coffee could also play a role in the anti-obesity effect of coffee, since 5-CQA has shown to decrease glucose absorption and modulate gastric hormone levels (Rodriguez de Sotillo and Hadley, 2002; Johnston et al., 2003; Thom, 2007), as well as to decrease the activity of lipoprotein lipase in skeletal muscle mediated by the regulation of PPAR-α expression in liver, thus facilitating lipid clearance and improving insulin sensitivity (Li et al., 2009). In overweight subjects who consumed a green coffee bean extract that contained high concentrations of chlorogenic acids, positive effects on glucose and fat metabolism were observed, as well as a significant reduction in body weight, body mass index and percent body fat (Vinson et al., 2012).

Consumption of coffee has been linked to a decreased risk of type 2 diabetes in spite of the clear evidences showing the increased hyperglicemic effect of caffeine and its effect increasing insulin resistance (Johnston et al., 2003; Tunnicliffe and Shearer, 2008). It has been postulated that 5-CQA decreasing glucose absorption would be the mechanism to counteract the hyperglycemic action of caffeine. Recently, it has been shown that mate is able to down-regulate SGLT1 (sodium-dependent glucose transporter) expression, the main glucose transporter in the intestinal epithelium, which would explain the effect of chlorogenic acid on glucose absorption and its contribution to the glucidic homeostasis (Oliveira et al., 2008b). Also, it has been hypothesized the effect of hydroxycinnamic acids decreasing carbohydrate digestibility and, therefore, decreasing the glycemic index, since the inhibitory action of 5-CQA on pancreatic alpha-amylase has been shown in *in vitro* studies (Narita and Inouye, 2009). There are some studies that point to an inverse association between coffee intake and diabetes, so that subjects who drank at least seven cups of

coffee a day were only 0.50 times as likely as those who drank two cups, or fewer, a day to develop this disease (van Dam and Feskens, 2002). Similar results have been described among Japanese health check-up examinees (Isogawa et al., 2003). In contrast, other findings suggest that coffee drinking provides no protection against T2DM (Reunanen et al., 2003).

Cancer

There is controversy in epidemiolgical studies that link mate intake with several types of cancer (oral cavity, pharynx, larynx, oesophagus, lung and bladder). Some studies show that high mate intake is associated with increased risk of oroesofagic cancer, being higher the risk when consumed with tobacco and alcohol (Pintos et al., 1994; De Stefani et al., 2003 Goldenberg, 2002; Goldenberg et al., 2003; Sewram et al., 2003). Other studies have not shown such association or only when mate is consumed very hot, due to the thermal injury of orobucal mucosa resulting more susceptible to mutagens and procarcinogens in mate, tobacco, etc. (Castellsague et al., 2000; Loria et al., 2009). A mutagenic and genotoxic effect has been shown for mate in the Ames test only in the absence of S9 microsomal fraction (Leitao and Braga, 1994; Fonseca et al., 2000), suggesting an important role of ROS on the mutagenic and potential carcinogenic effect of mate. Similarly, the high polycyclic aromatic hydrocarbons in mate (Kamangar et al., 2008) could also be partly responsible for such procarcinogenic effect of roasted mate, although recently it has been suggested that this genotoxic effect could be due to caffeine (Wnuk et al., 2009). In a bladder cancer case-control study, mate consumed with "bombilla" for 20 years was associated with bladder cancer in ever-smokers, but not in never-smokers. In contrast, mate “cocido” was not associated with bladder cancer. These results are consistent with a previous study in Uruguay (Bates et al., 2007). In cell model studies, mate leaves inhibited by 50% the growth of human colorectal adenocarcinoma Caco-2 and HT-29 cells when compared with the CCD-33Co non-cancer colon fibroblast cell line (de Mejía et al., 2010). Antiproliferative effects of mate phenolic compounds against oral cancer cells have been described consisting in inhibiting topoisomerases I and II (González de Mejía et al., 2005), and increasing apoptosis and necrosis in human lymphocytes (Wnuk et al., 2009). Similarly, matesaponins induced apoptosis through suppression of Bcl-2 and increased Bax protein expression and activated caspase-3 activity in HT-29 cells (Puangpraphant et al., 2011a). Toxicity studies

have been carried out with a yerba mate dried extract in rats and rabbits during 90 days; the extract showed no effects on survival, clinical observations, macroscopic examination of organs, body weight, or food and water consumption. Thus it was concluded that yerba mate extract does not promote toxic effects in the mammalian species studied (de Andrade et al., 2012).

On the other hand, coffee consumption has shown a decreased risk for several types of cancers (endometrial, liver, colon) (Lee et al., 2007; Shimazu et al., 2008; Inoue et al., 2009), although a recent study in Japanese subjects associates coffee and/or caffeine consumption with a potential increased risk of bladder cancer (Kurahashi et al., 2009). Protective effects against H_2O_2–induced DNA damage in liver, kidney and bladder cells isolated from mice treated with mate has been described (Miranda et al., 2008), an effect also observed in human colon cancer cells (HT29) after incubation with chlorogenic acid, green coffee extract or an extract of bread supplemented with green coffee antioxidants (Glei et al., 2006). The effect reducing cell viability and inducing apoptosis in tumour cells after treatment with hydroxycinnamic acids (Miccadei et al., 2008) could be associated to their antioxidant capacity, increasing the activity and expression of antioxidant enzymes in lung and epidermal tumor cells and suppressing cellular signals involved in cell survival and proliferation (NF-κB, activator protein-1 (AP-1), and MAPK) (Feng et al., 2005), although in a human liver cancer cell model such effect was not observed (Granado-Serrano et al., 2007)

Other studies have reported inconsistent findings on the association between coffee consumption and different types of cancer such as pancreatic, lung and breast cancer. In Japan, coffee consumption did not have a substantial impact on pancreatic cancer risk (Luo et al., 2007), in agreement with the results of a meta-analysis of coffee consumption and pancreatic cancer that indicated that coffee consumption was not appreciably related to pancreatic cancer risk, even at high intakes (Turati et al., 2012). However, this research group observed that an increase in coffee consumption of 2 cups/day was associated with a 14% increased risk of developing lung cancer (Tang et al., 2010). Accordingly, in another meta-analysis, high or increased consumption of coffee was related to an increased the risk of lung cancer (Xie et al., 2016). Heavy coffee consumption also showed an increased risk of breast cancer according to another meta-analysis (Tang et al., 2009).

2.3 Use of cell culture models to study the biological effects of dietary polyphenols.

2.3.1. Oxidative stress and cellular antioxidant systems

Oxidative stress is caused by ROS, which act as radicals and can damage macromolecules such as DNA, proteins and lipids. ROS are widely recognized as one of the causes of the development of chronic disease and ageing (Figure 5). Oxidative stress plays a major role in many disorders including cancer, diabetes, Alzheimer's disease, stroke, viral infections, neurodegenerative processes, infarction, brain oedema, and also aging (Krishnamurthy and Wadhwani, 2012). Dietary antioxidants protect against free-radical reactions in human disease, their bioavailability and toxicology are topics of interest although there is certain controversy. Antioxidant activity is one of the most important biological properties of phenolic compounds (El Gharras, 2009), which act as free radical scavengers, electron or hydrogen donors, and strong metal chelators, preventing lipid peroxidation, DNA damage and other macromolecules (Pandey and Rizvi, 2009).

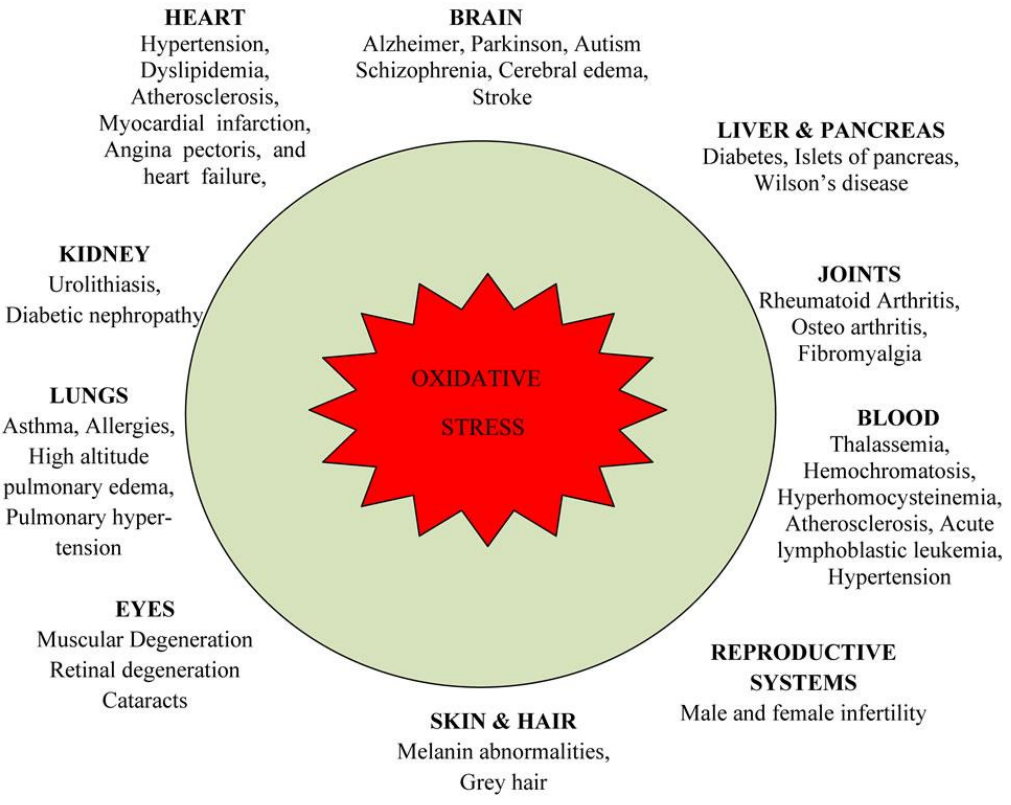


Figure 5. Oxidative stress relate to human diseases. Source: Rahman et al., 2012.

Cells possess a defence system that regulates the expression and levels of a number of antioxidant enzymes acting as a defence mechanism of protection against the damage induced by free radicals. In this sense, cells of living organisms have two major defence mechanisms against free radical damage: non-enzymatic (reduced glutathione and dietary antioxidants such as vitamin E) and enzymatic (superoxide dismutase, glutathione peroxidase and catalase) (Rahman et al., 2012). Reduced glutathione (GSH) is the main non-enzymatic antioxidant endogenous defence functioning as a substrate in glutathione peroxidase-catalysed detoxification of organic peroxides (Figure 6), through reacting with free radicals and repairing free radical induced damage in electron-transfer reactions. GSH directly quenches ROS such as those formed in lipid peroxidation, and also plays a major role in xenobiotic metabolism. The main protective roles of GSH against oxidative stress are: (i) it can act as a co-factor for several detoxifying enzymes, (ii) participates in amino acid transport across plasma membrane, (iii) scavenges hydroxyl radical and singlet oxygen directly, and (iv) regenerates vitamins C and E back to their active forms (Masella et al., 2005).

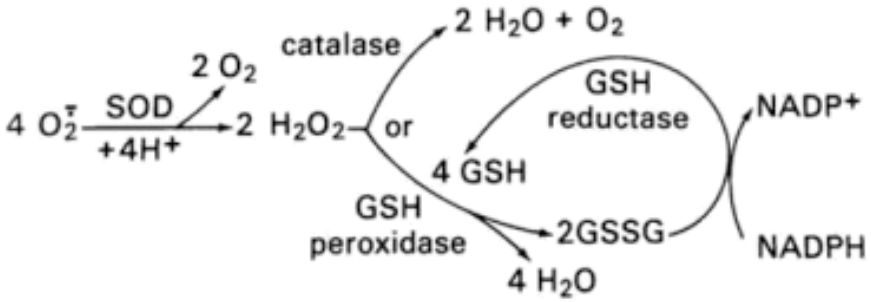


Figure 6. Major endogenous oxidative enzymatic reactions system.

Enzymatic defences are categorized into primary enzymatic [glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD)] and secondary enzymatic (glutathione reductase, GR) defences. Figure 4 shows the corresponding reaction system. Primary defences prevent the formation or neutralize free radicals. Glutathione peroxidase donates two electrons to reduce peroxides by forming selenols and also eliminates peroxides, catalase converts hydrogen peroxide into water and molecular oxygen and SOD converts superoxide anions into hydrogen peroxide as a substrate for catalase. Regarding the secondary enzymatic defence system, glutathione reductase reduces

glutathione from its oxidized (GSSG) to its active reduced (GSH) form, thereby being recycled to continue neutralizing more free radicals (Krishnamurthy and Wadhwani, 2012).

2.3.2. Human cell models to assess effects against oxidative stress

Human cell models are widely used to study the polyphenols' protective effects against oxidative stress. The different cell models allow studying both chemoprevention and molecular mechanisms of action of phenolic compounds by simulating an oxidative stress condition. The different cell lines are obtained from main target tissues in diseases associated with oxidative stress, such as liver, pancreas, colon, adipose tissue or skeletal muscle.

Many phenolic chemoprevention studies focus on the response of liver cells, since liver is the main site for xenobiotic metabolism. **The human hepatoma cell line HepG2** is considered a good model to study *in vitro* xenobiotic metabolism and toxicity to the liver, since these cells retain many of the specialized functions which characterize non-cancer human hepatocytes (Knasmuller et al., 1998). This model has also been extensively used in biochemical and nutritional studies where many compounds and conditions have been assayed with minor interassay variations (Goya et al., 2009). Many phenolic compounds such as luteolin, quercetin, rosmarinic acid, luteolin-7-glucoside or caffeic acid, among others protect HepG2 cells against from oxidative damage induced by *tert*-butylhydroperoxide (*t*-BOOH) (Lima et al., 2006; Goya et al., 2009). Several studies have shown that different flavonoids, thanks to their chemical structure, reduce oxidative stress through numerous mechanisms. For example, flavanols, such as epicatechin, reduced oxidative stress by recovering GSH levels, decreasing ROS and muffling the increase of GPx and GR in human HepG2 cells after exposure to *t*-BOOH (Martin et al., 2010a). Quercetin (1-100 μM) and rutin (0.1-100 μM) diminished ROS production and at higher doses (10–100 μM) of quercetin and rutin decreased the concentration of the lipid peroxide biomarker MDA. Furthermore, rutin at 100 μM induced favourable changes in GR levels in HepG2 cells that prevented or delayed conditions which favoured cellular oxidative stress (Alia et al., 2006a). In another study carried out in HepG2 cells exposed to *t*-BOOH, quercetin (0.1–10 μM) prevented the decrease of GSH and the increase of MDA, GPx, SOD, GR and catalase activities induced by *t*-

BOOH (Alia et al., 2006b). At the molecular and oxidative mechanism levels in HepG2 cells, flavonoids in a cocoa phenolic extract activated survival signalling proteins, such as protein kinase B (PKB/AKT) and extracellular signal-regulated protein kinases (ERKs) and increased the activities of GPx and GR to protect against oxidative stress-induced apoptosis (Martin et al., 2010b).

In a study using a green coffee bean extract (GCBE) rich in hydroxycinnamic acids (84% of the total phenolic content) at concentrations 1, 10 and 50 μg/mL, the extract counteracted the damage induced by *t*-BOOH in HepG2 cells, returning GSH levels and the activity of both antioxidant enzymes, GR and GPx, to values similar to those found in control cells, in addition to reducing protein and lipid oxidation damage (Baeza et al., 2014). Similarly, a yerba mate phenolic extract (1, 10 and 50 μg/mL) showed protective antioxidant effects recovering GSH levels, and GPx and GR activity, decreasing macromolecular damage by reducing MDA and carbonyl groups concentrations (Baeza et al., 2016).

In addition to studying the antioxidant effects of the green coffee and yerba mate extracts, their main phenolic components 5-CQA and 3,5-diCQA at concentrations 1, 10, and 20 μM were evaluated and showed similar behaviour against oxidative stress *in vitro*, reversing the *t*-BOOH induced increase in GPx and GR levels and preventing macromolecular damage of both proteins and lipids (Baeza et al., 2014). Moreover, the antioxidant effects of the major colonic microbial metabolite of CGAs, dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids were studied again in HepG2 cells. DHCA at 0.2, 1, 10 μM prevented cytotoxicity and macromolecular damage induced by *t*-BOOH, in contrast to DHFA which showed a slight protection against cell cytotoxicity, lipid oxidation and GSH depletion (Baeza et al., 2016).

Virgin olive oil phenols, hydroxytyrosyl acetate (HTy-Ac) and hydroxytyrosol (HTy), have also shown antioxidant stress protective effects at physiological concentrations and counterbalanced GSH levels, protected against lipid peroxidation and decreased ROS generation in HepG2 cells (Pereira-Caro et al., 2012a).

Intestinal epithelium is exposed to toxicity induced by luminal oxidants from food after consumption, which can cause oxidative damage to macromolecules and tissues. This fact supports the interest of studying oxidative

stress-induced toxicity and the effect of dietary antioxidants in the intestinal epithelium. **Human Caco-2 cell line**, derived from human colon cancer cells, is also widely used for biochemical and nutritional studies as a cell culture model of human colonocytes, since it retains their morphology and most of their function in culture (Rodriguez-Ramiro et al., 2011b). Under certain culture conditions, Caco-2 cells can form spontaneously polarized microvillus, closely connected with differentiation characteristics mimicking intestinal epithelium (Artursson and Karlsson, 1991; Yamashita et al., 2000). For this reason, Caco-2 cells have been used to study the intestinal absorption of food components such as peptides (Amigo-Benavent et al., 2014) and phenolic compounds (Konishi and Kobayashi, 2004; Mateos et al., 2011; Gallardo et al., 2016), among other dietary ingredients. This cell model has also been used to test oxidant-induced stress related intestinal injury and gut pathologies. The flavanols epicatechin, epicatechin-3-gallate, epigallocatechin-3-gallate and procyanidin B2 protected Caco-2 cells against induced oxidative stress and subsequent cellular death by reducing ROS production and preventing caspase-3 activation (Rodriguez-Ramiro et al., 2011a). In particular, procyanidin B2 increased the activity of antioxidant/detoxification enzymes and thus protected Caco-2 cells (Rodriguez-Ramiro et al., 2011a). When the molecular mechanisms involved were studied, procyanidin B2 was found to protect against oxidative stress in colonic cells by up-regulating the expression of glutathione S-transferase (GST) via a mechanism that involves ERKs and p38 MAPK activation and NF-erythroid 2-related factor (Nrf2) translocation (Rodriguez-Ramiro et al., 2012).

Acrylamide is a compound present in certain cooked foods, and there have been concerns prompted about the carcinogenicity induced by this compound. When acrylamide oxidative stress effects were induced in Caco-2 cells, olive oil HTy at concentrations between 5 and 40 μM reduced cell toxicity and ROS generation, recovering enzyme antioxidant defences and decreasing phospho-Jun kinase (p-JNK) concentration and caspase-3 activity (Rodriguez-Ramiro et al., 2011b).

Oxidative stress is accepted as one of the causes of pancreatic beta cell failure in type 2 diabetes. Chronic high glucose exposure would directly increase intracellular ROS generation and deteriorate mitochondrial function to uncouple ATP generation, impairing the glucose-stimulated insulin secretion (Martin et al., 2014). In this context, **Ins-1E pancreatic beta cells** are used as another

oxidative stress cell model. Cocoa phenolic extracts counteracted oxidative stress in Ins-1E cells by preventing the *t*-BOOH-induced ROS production, lowering the levels of carbonyl groups as a protein oxidation biomarker, and returning antioxidant defences to adequate levels (Martin et al., 2013). Regarding the mechanisms involved, epicatechin protected pancreatic beta cell viability and oxidative status, via the down regulation of p-JNK expression and restored insulin secretion damaged by *t*-BOOH (Martin et al., 2014). Moreover, pre-treatment with microbial flavonoid metabolites 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid also prevented cell dysfunction and death induced by *t*-BOOH through the activation of protein kinase C (PKC) and ERK pathways in this beta cell line, Ins-1E (Fernandez-Millan et al., 2014).

There are also many other cell models of oxidative stress used to study the pathogenesis of brain disorders and injury in the central nervous system. Oligodendroglial, astroglial, microglial cells and neurons demonstrated prominent differences in pro-oxidant detoxication capacities, with oligodendroglial cells in culture having the most prominent machinery for ROS disposal, which is likely to support the protection of oligodendrocytes *in vivo* in the brain under oxidative stress conditions (Hirrlinger et al., 2002). Five catechins [(-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), and (+)-catechin (C)] were compared with regard to their effects on 6-hydroxydopamine induced oxidative apoptosis in PC12 cells as an *in vitro* model of Parkinson's disease. Results indicated that those catechins have significant difference in the degree of antioxidant protection, and that EGCG and ECG might be potent neuroprotective agents for Parkinson's disease (Jin et al., 2001).

2.3.3. A human cell model to study effects on vascular function.

Vascular endothelium plays a central role in the regulation of vascular tone, by regulating local cellular proliferation, producing paracrine factors that act on the arterial wall and blood cells, and controlling homeostatic as well as inflammatory responses (Widlansky et al., 2003). Endothelial cells produce a variety of vasculoregulatory and vasculotropic molecules that act locally or at distant sites. Alteration of the vascular endothelium is a primary event in the pathogenesis of vascular diseases and is a critical target for preventing or

slowing the progression of CVD. Endothelial dysfunction is recognized as the initial step in the atherosclerotic process and is well described in patients with diabetes and atherosclerosis (Schalkwijk and Stehouwer, 2005). For these reasons, studying the human endothelium is relevant in cardiovascular research, as understanding the cellular and molecular biology of the endothelial cells is essential for the development of new approaches for both the prevention and therapy of CVD.

The **EA.hy926 cell line** is a human umbilical vein endothelial cell (HUVEC) line, derived from the fusion of HUVECs and lung adenocarcinoma cells. The structural characteristics and functions are similar to lung adenocarcinoma cells and these alveolar endothelial cells are used to construct the alveoli *in vitro* mouse model (Xu et al., 2008). EA.hy926 cells have been widely applied in the study of oxidative stress and protein expression, as well as leukocyte adhesion to endothelial cells (Emeis and Edgell, 1988).

Epidemiological studies suggest that dietary intake of polyphenol-rich foods and supplementation with bioactive components have protective effects against CVD (Dohadwala and Vita, 2009; Pandey and Rizvi, 2009; Wightman and Heuberger, 2015). The mechanisms involved in these properties mainly include regulation of lipid metabolism, attenuation of oxidative damage and scavenging of free radicals, improvement of the endothelial function and vascular tone, enhancement the production of vasodilating factors such as NO, and inhibition of the synthesis of vasoconstrictors such as endothelin-1 in endothelial cells (Pandey and Rizvi, 2009). Endothelial nitric oxide synthase (eNOS) plays a key role in the function of vascular endothelium. NO is produced by nitric oxide synthase (NOS) enzymes, of which there are three isoforms encoded by separate genes: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). eNOS is predominantly expressed by tumour vascular endothelial cells when stimulated by inflammatory cytokines and can produce NO (Huang, 2003). NO is an important signaling molecule that essentially regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Naseem, 2005).

Figure 7 illustrates the production of NO in tumours. Inflammatory cytokines interferon (IFN)-γ, IL-1β and TNF-α induce iNOS expression through

NF-κB. Hypoxia also induces iNOS through hypoxia-inducible factor 1α (HIF1α), whereas wild-type tumour suppressor p53 inhibits iNOS expression, mutant p53 fails to do so. Angiogenic factors such as vascular endothelial growth factor (VEGF), sphingosine-1-phosphate (S1P), angiopoietins, sex hormones, and shear stress activate eNOS in vascular endothelial cells through adenylate cyclase (AC)–protein kinase A (PKA), phosphoinositide-3-kinase (PI3K)–Akt, phospholipase Cγ (PLCγ) – diacylglycerol (DAG)–protein kinase Cα (PKCα) and PLCγ–cytosolic calcium (Ca^{2+})–calmodulin (CaM) pathways. Metabolic stress also activates eNOS through AMP kinase (AMPK). NO is produced by all these different sources in tumours (Fukumura et al., 2006).

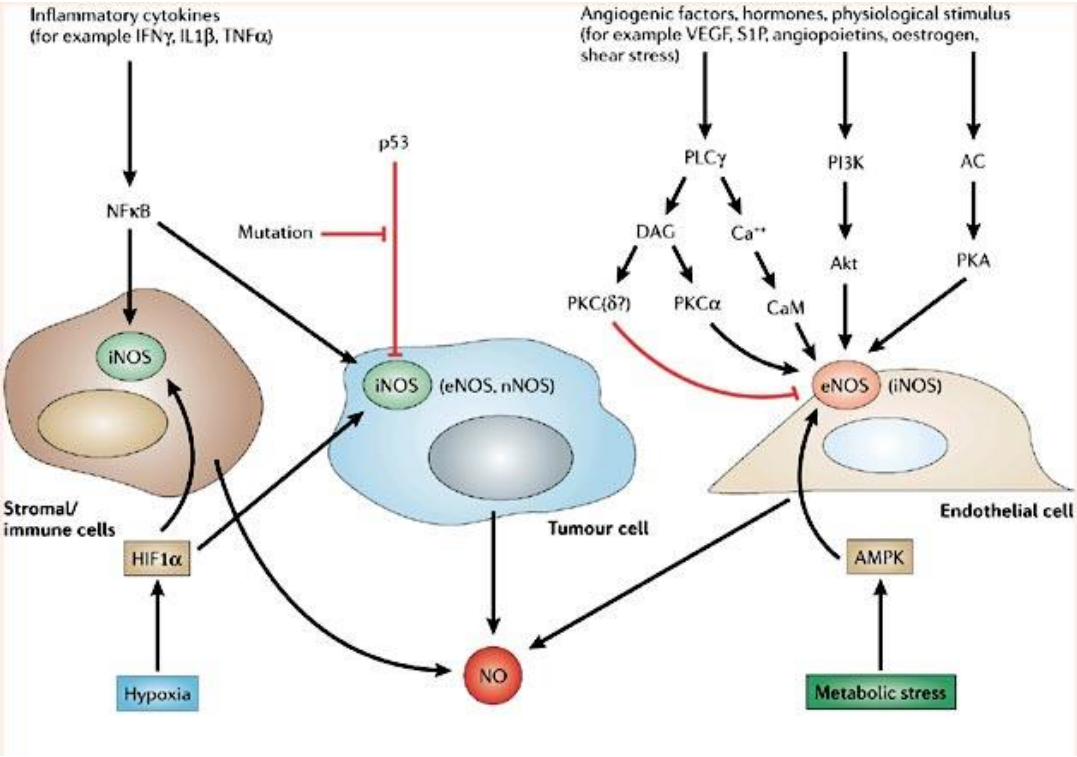


Figure 7. Production of nitric oxide (NO) in tumours (From Fukumura et al., 2006)

Procyanidins, monomeric flavan-3-ols, flavonols, flavones, flavanones, chalcones, stilbenes, and phenolic acids have been tested to understand their ability to enhance endothelial NO levels. The results showed that resveratrol, quercetin, ECG, and EGCG might enhance NO levels by 285%, 110%, 85%, and 60% respectively with an increased amount of eNOS enzyme as a possible contributing mechanism by using the hybrid human endothelial cell line EA.hy926 (Appeldoorn et al., 2009). Chlorogenic acid also exhibited anti-inflammatory

effects in HUVECs by inhibiting of U937 monocyte-like adhesion, adhesion molecule expression, NF-*κ*B translocation and ROS production (Chang et al., 2010). Quercetin attenuates monocyte chemoattractant protein-1 (MCP-1) expression in high glucose treated rat aortic endothelial cells, probably by regulating both NF-*κ*B and AP-1 pathways, indicating the potential in abating the risk for atherosclerosis (Panicker et al., 2010). The flavonoids luteolin and cynaroside from artichoke (*Cynara scolymus* L.) increased eNOS promoter activity and eNOS mRNA expression in human endothelial cells, with lipid-lowering and antioxidant properties (Li et al., 2004).

2.3.4. Human cell lines to evaluate antiproliferative effects

According to the World Health Organization (WHO), cancer is the uncontrolled growth of cells, which can invade and spread to distant sites of the body. The uncontrolled growth and proliferation can be initiated by an early mutation or epigenetic change as a consequence of external growth factors, signals or mutations to DNA. Such changes however are not the only mechanisms for increasing cell number, the inactivation of programmed cell death, also known as apoptosis, contribute to the growth and proliferation of a tumour following the steps summarize in Figure 8 (Alberts et al., 2008). Therefore uncontrolled cell proliferation, considers the increase in the number of cells as a result of growth and division and it is a primary characteristic of cancer cells (Kampa et al., 2007).

The improvement of cancer control, prevention and therapeutic has decreased cancer-related deaths by 20% in the past 20 years (Siegel et al., 2014). Identifying dietary strategies to control the proliferation of cancer cells may form part of a prevention therapy against the onset and progression of cancer. Diets rich in fruits and vegetables have shown a protective effect against many cancers (http://www.who.int/cancer/prevention/en/), Different animal and cell studies have demonstrated that phenolic compounds can inhibit cell proliferation, reduce the number and growth of tumours and thus may be useful as potential chemopreventive agents against cancer. Although the mechanisms by which phenolic compounds can have a protective effect against cancer are not fully understood, they have shown to act on multiple key elements such as in signal transduction pathways related to cellular proliferation, differentiation, apoptosis,

inflammation, angiogenesis and metastasis (Ramos, 2008). At first, phenolic compounds may act as blocking agents at the initiation stage; they can form potentially toxic quinones that are substrates of phase II conjugating enzymes being toxic for cancer cells. Secondly, phenolic compounds can also stimulate DNA repair and thus prevent the modified changes that lead to a cancer cell. Thirdly, phenolic compounds can act as suppressing agents of tumours from initiated cells by inhibiting cell proliferation or inducing apoptosis (Scalbert et al., 2005).

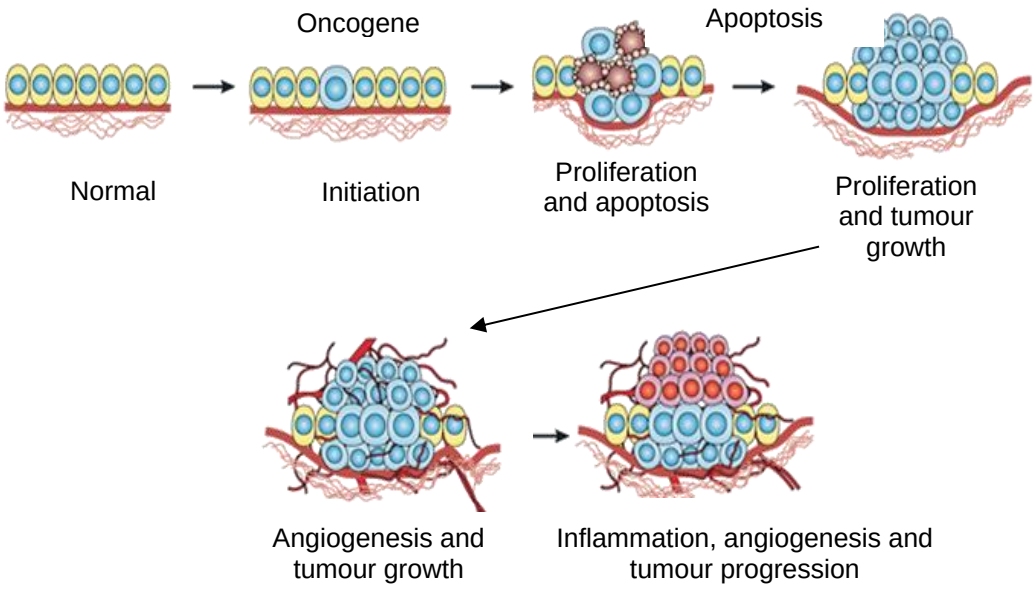


Figure 8. Diagram of tumour initiation and cancer progression. Modified from Mathew et al., 2007.

Cancer cell lines provide excellent models for studying the physiology and biochemistry of cancer, as well as their metabolic and signalling pathways, allowing studying the effect of phytochemicals, drugs and toxic compounds. There are numerous studies that have employed phenolic compounds or food extracts rich in polyphenols in cancer lines to study their cytotoxicity and antiproliferative effects, some examples are shown in Table 7. Ideally, a good antiproliferative compound would be one with high cytotoxic or antiproliferative effects on cancer cells but not on healthy ones.

Table 7. Examples of studies with cancer cell lines and phenolic compounds.

Cancer	Cell lines	Phenolic compound/extracts	References
Oral	OSCC-3 and SCC-61 human squamous carcinoma lines	(-)-epigallocatechin gallate	González de Mejia et al., 2005
Oesophageal	TE-2 oesophageal cancer	Gallic acid	Faried et al., 2007
Lung	A549 adenocarinoma SBC-3 small carcinoma Calu-6 lung carcinoma	Gallic acid	Subramanian et al., 2015
Colon	HT29 cells SW480	Caffeic, 5-caffeoylquinic acids Resveratrol	Murad et al., 2015 Joe et al., 2002
Cervical	HeLa cervical cancer	Green tea, coffee and cocoa extracts	Krstic et al., 2015
Bladder	Urinary bladder cancer T24 (HTB-4)	Ferulic acid	Peng et al., 2013
Liver	HepG2	Chlorogenic acid, quercetin, epicatechin	Ramos et al., 2005

Human oesophageal carcinoma cell lines (OE-33) has been used as a model of oesophageal cancer. OE-33 together with other carcinoma esophageal cell lines (OE19, OE21) have been used in mechanistic studies to unravel the mechanism of action of anticancerigen drugs (deferoxamine and deferasirox) against oesophageal cancer (Ford et al., 2013). OE-33 cells have also been used to study the antiproliferative effect on oesophageal adenocarcinoma of curcumin, which promotes apoptosis via caspases 3 and 7 (Hartojo et al., 2010). **Human lung carcinoma cell line (A549)** has been used as a lung cancer model. A549 cells have been treated with cocoa methylxanthines derivatives (Ruddarraju et al., 2016) and rosemary extracts (Moore et al., 2016), among other compounds to study their anticancer and antiproliferative effects. **Human urinary bladder (T24)** carcinoma cells have also been used to understand the antiapoptotic and antiproliferative effects of green tea catechins (Philips et al., 2009), piceatannol (Kuo and Hsu, 2008), and curcumin (Park et al., 2006). **Human HepG2 and Caco-2 cell lines,** apart from being a cell culture model of human hepatoma and human colorectal adenocarcinoma, respectively as indicated in section 2.3.2, they are tumour cells and therefore can be also employed as cancer lines in proliferation and cytotoxicity studies. Caco-2 cells have been used to study the antiproliferative effects of cocoa flavanols (Ramos et al., 2011), sulforaphanes

from broccoli sprouts (Baenas et al., 2015) or soybean peptides (Amigo-Benavent et al., 2014), among others, while HepG2 cells have been used to test the effects of flavonols (Granado-Serrano et al., 2010a), flavanols (Granado-Serrano et al., 2010b), hydroxycinnamic acids (Granado-Serrano et al., 2007) or cocoa and olive oil polyphenols (Martin et al., 2012).

Human CCD-18Co cells are non-malignant colonic fibroblast cells and have been employed as a model of colon cell line to study cytotoxic effects of soybean peptides (Clemente et al., 2010). Human CCD-33Co cell line is another example of normal colon fibroblast cell line which has been used to study the proliferation and cytotoxicity of yerba mate extracts (de Mejia et al., 2010) and dicaffeoylquinic acids (Puangpraphant et al., 2011a).

In vitro cell culture studies have shown that tea polyphenols have the potential to induce apoptotic cell death and cell cycle arrest in tumour cells but not in their normal cell counterparts (Chen et al., 2008). Green tea, coffee and cocoa extracts have also shown to induce apoptosis in human cervical adenocarcinoma HeLa cells (Krstic et al., 2015). In contrast, no significant correlations were found between antiproliferative activities and total phenolics or total flavonoid contents in *Vitis vinifera* grape cultivars (Liang et al., 2014).

Among flavonoids, quercetin is a potent inhibitor of tumour initiation in vivo and presents antiproliferative activities against liver tumour cells (HepG2) *in vitro* (Granado-Serrano et al., 2008). Many studies have been carried out to assess the anticancer effects of resveratrol. This phenolic compound has shown growth-inhibitory activity in several human cancer cell line (Khan et al., 2014). In HL60 promyelocytic leukemia cells, treatment with resveratrol led to growth inhibition, induction of apoptosis, S-G2-phase cell cycle arrest, and myelomonocytic differentiation (Clement et al., 1998; Ragione et al., 1998). In Caco-2 cells, 25 μM resveratrol caused a 70% growth inhibition (Schneider et al., 2000). This stilbene also induced growth inhibition, S-phase arrest, apoptosis, and decreased expression of cyclin B1 in Seg-1, Bic-1, SW480, and MCF7 cell lines whereas the expression of cyclin A, cyclin B1 and β-catenin changed in only in SW480 cells (Joe et al., 2002).

The inhibition of carcinogenesis by mate and coffee has been demonstrated in animal models in addition to many different cell culture experiments (Bracesco et al., 2011; Heckman et al., 2010; Nkondjock, 2009).

Yerba mate saponins inhibit human colon cancer HT-29 and RKO cell proliferation by inducing cell cycle arrest and apoptosis via p53 cascade (Puangpraphant et al., 2012). Caffeic and 5-caffeoylquinic acids were able to reduce HT-29 cell viability, promoting specific changes in the cell cycle and increasing the apoptosis rate, suggesting a modulation of the cell cycle with an increase in apoptosis (Murad et al., 2015).

Hypothesis and Objectives

Hypothesis and Objectives

The present work has focused on studying 3 products: a grape by-product, green coffee and a yerba mate tea. These products are sold as nutraceuticals at supermarkets, pharmacies and herbalist shops, and also can be sold and employed as functional food ingredients or beverages. All of them are rich in dietary polyphenols and consumers are demanding them to promote a healthier life style.

Grape or winery by-products can increased the value of grape use in wine producers. These by-products contain dietary polyphenols being phenolic acids, flavonoids (anthocyanins, catechins, procyanidins, flavonol glycosides, etc.), and stilbenes the most abundant. Observational and clinical studies have associated grape by-products with a decreased risk of CVD, a possible reduction in metabolic syndrome and a promising anticancer potential.

On the other hand, yerba mate leaves and green coffee beans are rich in hydroxycinnamic acids. Yerba mate is used for the preparation of tea-like beverage widely consumed in South America. Green coffee is sold as soluble powdered coffee mixed with roasted coffee consumed as a stimulating beverage, and also as a dietetic supplement. Both products have been related with benefits in cardiovascular function, obesity and type 2 diabetes, but there are contradictory reports on the effect of coffee and yerba mate consumption and an increased risk of certain types of cancer. Green, un-roasted coffee beans have been less studied than roasted coffee, although its richer phenolic content deserves further studies.

The **hypothesis** of this thesis is that grape by-products, green coffee beans and yerba mate leaves as phenolic-rich products may present positive biological properties resulting in beneficial health effects, protecting cells against oxidative stress, improving vascular function or avoiding proliferation of cancer cells. Thus, these products may be of relevance as functional ingredients or nutraceuticals aimed particularly at diseases associated with oxidative stress.

To prove this hypothesis, polyphenol-rich extracts from red grape pomace, green coffee beans and yerba mate leaves were obtained and their biological activities assessed in cell models of oxidative stress, cardiovascular function and cancer.

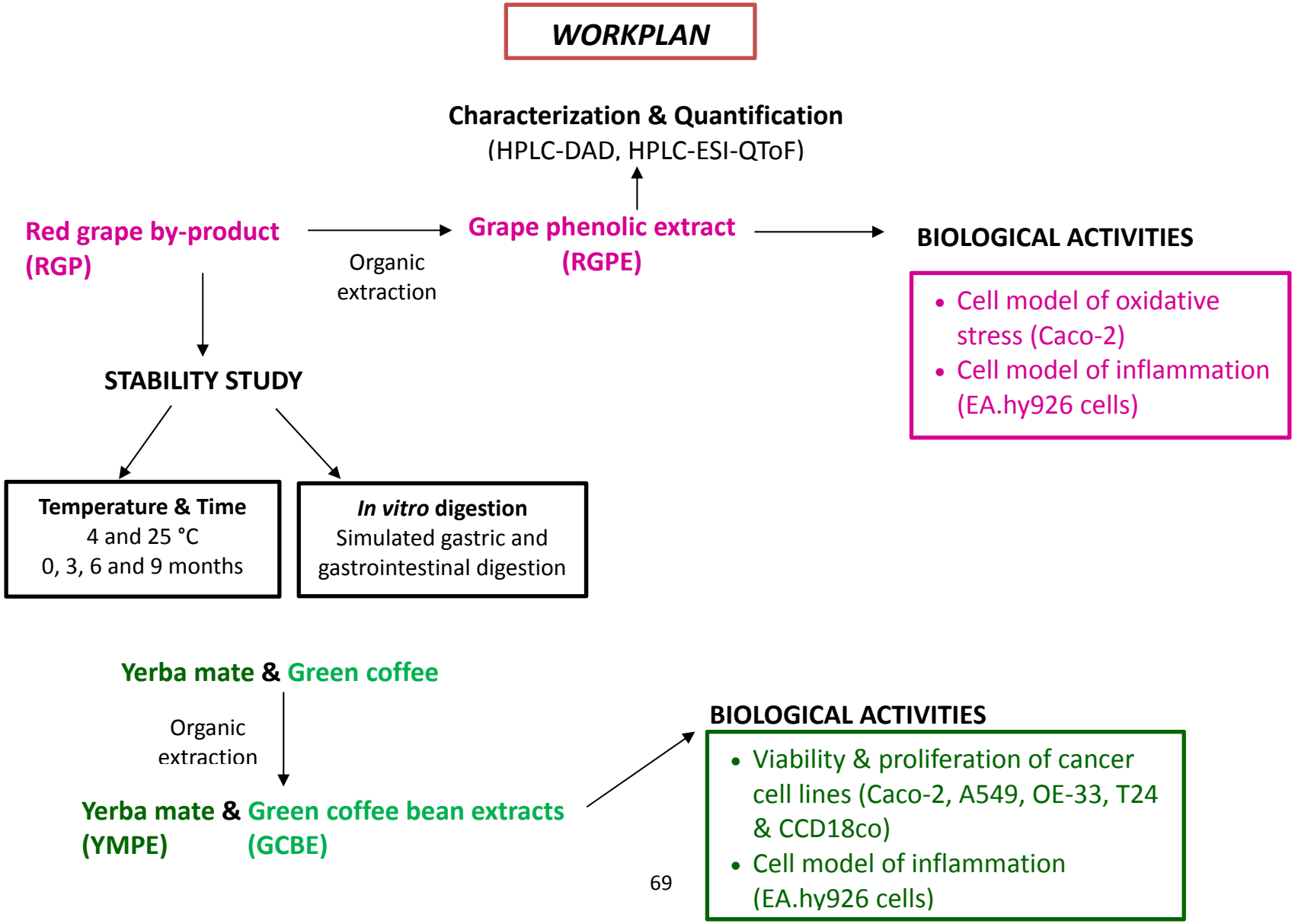
Some of these properties had already been explored in previous studies of the research team such as the effect of green coffee beans and yerba mate extracts on cellular oxidative stress (Baeza et al., 2014; 2016) after a comprehensive characterization of the phenolic composition of both extracts (Baeza et al., submitted a-c). This, however, was not the case of the grape by-product, which was specially intended as a potential functional food ingredient or nutraceutical. Therefore, characterization of this product and assessment of its stability under different storage conditions were preliminary steps in the assessment of its potential use as food ingredient.

Considering the previous hypothesis, the main objective of this Doctoral Thesis was to study some biological properties of the phenolic fraction of a red grape pomace, green coffee beans and yerba mate leaves.

To this aim, the following specific objectives were proposed:

1. Characterisation of a red grape pomace determining its phenolic composition, antioxidant capacity, as well as moisture, colour, and microbiological safety.
2. To study the stability of the red grape pomace during storage (4 and 25°C for up to 9 months) as well as the effect of *in vitro* gastrointestinal digestion.
3. Evaluation of the capacity of the red grape pomace phenolic extract and its phenolic components gallic and syringic acids to protect intestinal Caco-2 cells against an oxidative damage induced by *tert*-butylhyroperoxide.
4. Assessment of the capacity of red grape pomace, green coffee beans and yerba mate phenolic extracts and their main phenolic components and metabolites to protect human endothelial EA.hy926 cells against inflammation induced by TNF-α.
5. To study the antiproliferative effects of green coffee beans and yerba mate extracts, their main phenolic compounds and metabolites on different cancer cell lines.

To accomplish these objectives, the following work plan was proposed and developed.


WORKPLAN
Characterization & Quantification
(HPLC-DAD, HPLC-ESI-QToF)
Red grape by-product (RGP)
Organic extraction
Grape phenolic extract (RGPE)
BIOLOGICAL ACTIVITIES
• Cell model of oxidative stress (Caco-2)
• Cell model of inflammation (EA.hy926 cells)
STABILITY STUDY
Temperature & Time
4 and 25 °C
0, 3, 6 and 9 months
In vitro digestion
Simulated gastric and gastrointestinal digestion
Yerba mate & Green coffee
Organic extraction
Yerba mate & Green coffee bean extracts
(YMPE) (GCBE)
BIOLOGICAL ACTIVITIES
• Viability & proliferation of cancer cell lines (Caco-2, A549, OE-33, T24 & CCD18co)
• Cell model of inflammation (EA.hy926 cells)

Materials and Methods

4.1. Reagents and dietary products

4.1.1. Reagents

Ethanol, acetone, *n*-butanol, methanol, and 35% (v/v) hydrochloric acid for extraction were from Panreac (Madrid, Spain). Iron (II) sulfate-7-hydrate was acquired from Panreac (Sevilla, Spain). Iron (III) chloride hexahydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), 2,2'-Azobis(2-amidinopropane dihydrochloride (AAPH), pancreatin (P-1750), pepsin (77160), bile salts (B-8631), *tert*-butylhydroperoxide (t-BOOH), *o*-phthaldialdehyde (OPT), glutathione reductase (GR), reduced L-glutathione (GSH) and oxidized (GSSG) glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2,4-dinitrophenylhydrazone (DNPH), *β*-mercaptoethanol, guanidine, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), dimethylsulphoxide (DMSO), Sodium dodecyl sulphate (SDS), phosphoric acid, boric acid, acetic acid, sodium bicarbonate, and sodium pyruvate were purchased from Sigma-Aldrich (Madrid, Spain).

Phenolic standards: gallic acid, ellagic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, caffeic acid, ferulic acid, 5-caffeoylquinic acid, dihydrocaffeic acid, dihydroferulic acid, (–)-epicatechin, rutin, quercetin, and myricetin, as well as the methylxanthine caffeine, were from Sigma-Aldrich (Madrid, Spain). 3,5-Dicaffeoylquinic acid was from Phytolab (Vestenbergsgreuth, Germany).

For cell culture experiments, gentamicin, penicillin, streptomycin, non-essential amino acids (NEAA), McCoy's 5A medium, and minimum essential culture medium (MEM) were acquired from Sigma-Aldrich (Madrid, Spain). Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), DMEM-F12 and RPMI 1640 with glutamine culture media, were from Biowhitaker Europe (Lonza, Madrid, Spain). Cell proliferation ELISA 5-bromo-2'-deoxyuridine (BrdU) kit and cell viability reagent WST-1 were purchased from Roche Diagnostics (Barcelona, Spain). Bio-Rad protein assay reagent was from Bio-Rad Laboratories (Madrid, Spain). Recombinant murine TNF-α was from PreproTech

(Tebu-bio, Madrid, Spain). eNOS ELISA kits were from R&D Systems, Materlab (Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain).

All the reagents/culture media used for the microbiological analysis are specified in the corresponding section.

Water was prepared by purification with a Milli-Q water purification system (Millipore, Bedford, MA). All other reagents were of analytical or chromatographic grade.

Cell lines: human lung carcinoma A549, human colorectal adenocarcinoma Caco-2, human oesophageal carcinoma OE-33, human urinary bladder carcinoma T24, non-cancer fibroblastic cell line CCD-18Co were supplied by the Cell Bank of the Scientific Instrumentation Centre at the University of Granada (CIC-UGR, Granada, Spain). Human umbilical vein cells- EA.hy926 cells were kindly provided by Centro de Investigaciones Biológicas (CIB-CSIC, Madrid, Spain).

4.1.2. Dietary products

4.1.2.1. Grape by-product

The red grape by-product (RGP) (Figure 9a) employed was provided by a winery from the D.O. Ribera del Duero, Spain. The sample was a dry powder of red grape pomace obtained from grapes (*Vitis vinifera* L.) Tempranillo variety from vineyards in the Ribera del Duero region (Valladolid, Spain). All information on previous treatments of the grape by-product to obtain the final dried powder, initially designed to be used as a nutraceutical or food ingredient, is under industrial property protection.

4.1.2.2. Green coffee

Green coffee (*Coffea arabica* L.), from Colombia, was purchased in a local supermarket in Madrid (Spain) (Figure 9b) Due to their hardness, the green coffee beans were milled first in a domestic coffee grinder and then in a mill to a final particle size of 0.5 μm.

4.1.2.3. Yerba mate

Yerba mate (*Ilex paraguariensis* St. Hil.) tea was obtained in a local supermarket in Madrid (Spain). It contained dry leaves from mate, together with some stems (Figure 9c). Extracts from the mate tea were obtained from the commercial product without further milling or selection of the leaves.

a b c

Figure 9. Grape by-product (a), green coffee beans (b) and yerba mate (c).

4.2. Extraction of phenolic compounds from dietary products

The phenolic compounds were extracted from the different samples (red grape by-product, green coffee beans and yerba mate) in triplicate by organic solvent extraction following the protocol described by Bravo and Saura-Calixto (1998). Briefly, 1 g of sample was mixed with 40 mL of 2N HCl in aqueous methanol (50/50, v/v) using an orbital shaker at room temperature for 60 min, and centrifuged (3000 *g*, room temperature, 10 min). The supernatant was collected and the pellet was re-extracted with 40 ml of 70% (v/v) acetone by orbital shaking at room temperature for 60 min and centrifuged (3000 *g*, room temperature, 10 min). The supernatant was collected and the final pellet was dried at 60°C overnight to determine condensed tannins (section 4.3.1.2). The extracts **[grape phenolic extract (GPE), green coffee bean extract (GCBE) and yerba mate extract (YME)]** were obtained by combining the corresponding supernatants from both extractions and making them up to 100 mL. The extracts were aliquoted and stored at -20 °C for further use.

4.3. Analysis of the grape by-product

The grape by-product used in the present Thesis was analysed for its phenolic composition and antioxidant properties. Also, the effect of prolonged (up to 9 months) storage of the RGP at different temperatures on the stability and antioxidant properties of the RGP polyphenols was determined, as well as the effect of *in vitro* gastrointestinal digestion in an attempt to assess the suitability of this product as a functional food ingredient/nutraceutical.

It has to be pointed out that the analysis and antioxidant properties of the GCBE and YME were not performed in this Thesis, since both plant materials had already been extensively characterized by the research team in previous studies/thesis.

4.3.1. Identification and quantification of phenolic compounds in GPE

4.3.1.1. Quantification of total phenolic compounds

Total phenolic compounds of GPE were determined by the Folin-Ciocalteau colorimetric assay (Box, 1983). The assay was performed by mixing 0.5 mL of GPE and 0.5 mL of Folin-Ciocalteau reagent for 3 min; then 10 mL Na_2CO_3 solution (75 g/L) were added and the volume bought to 25 mL with distilled water, mixed and incubated for 60 min. at room temperature protected from light. The absorbance of samples was measured at 750 nm in a Beckman DU640 spectrophotometer (Fullerton, CA, USA). A gallic acid calibration curve (50-200 ppm) was used. Results were expressed as mg gallic acid equivalents (GAE) per 100 g of dry matter (dm). Samples were determined by quadruplicate.

4.3.1.2. Characterization and quantification of phenolic compounds by HPLC–ESI-QToF and HPLC-DAD

The GPE was evaporated to dryness using a rotary evaporator (Strike, Steroglass, Perugia, Italy) to eliminate methanol, acetone and HCl, and freeze-dried (Telstar, Madrid, Spain).

For HPLC analysis, GPE was resuspended in 1% (v/v) formic acid and filtered through a PVDF 0.45 μm filter prior to injection on the HPLC system. An

Agilent 1200 series liquid chromatograph system coupled to an Agilent 6530A accurate-mass quadrupole time-of-flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies, Waldrom, Germany) was used. Separation was performed on a Superspher 100 RP18 column (250 mm × 4.6 mm i.d., 4 µm, Agilent Technologies) preceded by an ODS RP18 guard column in thermostatic oven at 30 ^{o}C. Each sample (20 µL) was injected and separated by using a mobile phase consisting of water (phase A) and acetonitrile (phase B), both containing 1% formic acid, at a flow rate of 1 mL/min. The solvent gradient changed from 10 to 20% phase B over 5 min, 20 to 25% phase B over 20 min, 25 to 35% phase B over 10 min, then maintained isocratically for 25 min and returning to the initial conditions over 10 min. Chromatograms were recorded at 280, 360 and 520 nm according to the expected maxima spectra of phenolic compounds. The Q-ToF acquisition conditions were as follows: 2 GHz, mass range between 100 and 1000 m/z, drying gas volume and temperature 10 L/min and 350°C, sheath gas volume and temperature 11 L/min and 350°C, nebulizer pressure 45 psi, cap voltage 3500 V for negative polarity and 4000 V for positive polarity, nozzle voltage 1000 V, and fragmentor voltage 75 V. Negative polarity was used to identify phenolic acids and flavonols, and positive polarity was used for anthocyanins. Data acquisition and qualitative analysis were performed by using MassHunter Data Acquisition Workstation Software (Agilent Technologies).

Phenolic composition of GPE was also analysed using an Agilent 1200 liquid chromatographic system equipped with an autosampler, quaternary pump and diode-array (DAD) detector. Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above. Chromatograms were acquired at 280 nm, 360 nm and 520 nm to quantify phenolic acids, flavonols and anthocyanins, respectively. Quantitative analysis was carried out by using the external standard method. Calibration curves of standard compounds were constructed by serial dilutions of commercial available phenolic compounds (section 4.1.1) dissolved in 10% DMSO at 10 mM (except ellagic acid, which was dissolved in 0.1 M NaOH) and injected onto the HPLC system. All analysis were done in triplicate and results expressed as µg/g dm as the mean value ± standard deviation.

4.3.2. Study of the stability of grape phenolic compounds

4.3.2.1. Storage of a grape by-product at different conditions

To carry out the stability test, the powdered RGP was split in batches of 50 g, sealed under vacuum with nitrogen gas in plastic tubes and stored in darkness at 4ºC and 25ºC for 0, 3, 6 and 9 months. The following parameters were determined at each time point: pH, colour, total phenolic compound content, moisture, condensed tannins, antioxidant capacity and microbiological analysis.

pH was determined as follows: RGP was dissolved at a concentration of 10 mg/mL in milliQ water with a magnetic stirrer at room temperature for 10 min and the pH measured by using a pH meter (Crison micro pH 2001, Madrid, Spain). The determinations were carried out by quadruplicate and results were expressed as mean ± standard deviation.

Moisture was determined following the official AOAC (1999) procedure. Briefly, 3 g of RGP were weighed in glass tubes and dried in the oven at 105°C for 3 h. After dryness, the samples were weighed again, and the moisture was calculated as percentage of moisture following equation (1):

Equation [1] $$\% \text{ Moisture} = [(W_g+W_s)-W_f]/ W_s \times 100$$

Where W_g is the weight of the glass tubes and the sample before the incubation in the oven, W_s is the weight of the sample, and W_f is the weight of the glass tubes and the sample after drying.

Colour of the samples was determined by covering a 30 mm diameter plate with RGP and using a colorimeter Konica Minolta CM-3500D for solids, assisted by software Spectramagic NX (Konica Minolta, Osaka, Japan). Colour was expressed following CIELAB scale (León et al., 2006). CIELAB scale employs a coordinate system L*, a* and b*; where L* value is the vertical coordinate of a three-dimensional system of colours, with values ranging from 0 (black) to 100 (white); a* is the horizontal coordinate with values ranging from –80 (green) to +80 (red); and b* is the third coordinate with values ranging from –80 (blue) to +80 (yellow). Colour was determined by quadruplicate.

Condensed tannins were extracted from pellets obtained from the extraction of phenolic compounds (section 4.2.1) using the HCl-butanol (5:95, v/v) method (Reed et al., 1982). The procedure was as follows: 50 mg of pellet was weighted in glass tubes with lid, 10 mL of HCl-butanol were added and the mixture was put into a water bath at 100°C for 3 h. After cooling, the samples were filtered through a number 2 tared Whatman filter-plate and brought to a volume of 25 mL with HCl-butanol. This corresponded with the condensed tannins solution. The tared filter-plate was dried with retained sample at 105°C overnight and weighted after cooling. Lastly, the absorbance of the condensed tannin solution was read at 553.5 nm wavelength against blank of HCl-butanol solution in a Beckman DU640 spectrophotometer. Condensed tannin content was calculated using a calibration standard curve of condensed tannins from carob bean (*Ceratonia silgua*, Nestlé).

Microbiological analyses were conducted following the corresponding procedures of the International Organization for Standardization (ISO) and European Normative (EN) directives to determine total mesophilic aerobic bacteria (ISO 4833), total *Enterobacteriaceae* (ISO 21528-2:2004), *Escherichia coli* (ISO 16649-2:2001), yeasts and moulds (NF V08-059), and *Salmonella* spp. (ISO 6579:2002).

RGP (10 g) was aseptically collected in a vertical laminar flow cabinet (Telstar AV-30/70) and homogenized into a Stomacher® bag with 90 mL of sterile buffered peptone water (Cultimed) using a Stomacher® 400 blender (Seward, Worthing, UK) for 1-2 min. Serial decimal dilutions were prepared in buffered peptone water and microbiological analyses were carried out as follows:

- **Mesophilic aerobic bacteria** were determined by plating 1 mL of the serial decimal dilutions on sterile agar medium plate count agar (PCA, Cultimed) and incubating in an oven at 30 ± 2 °C for 72 ± 2 h. The counting was performed on plates on which there was a colony growth between 0 and 300 Colony Forming Units (CFU) and the results were expressed in CFU/g of sample.
- **Total *Enterobacteriaceae*** were quantified after plating 1 mL of the serial decimal dilutions of the RGP on Violet Red Bile Agar (VRBA) petri dishes (Cultimed) and incubating in an oven at 37 ± 2 °C for 48 ± 2 h. The counting

was performed on plates on which there was a colony growth between 0 and 300 CFU and the results were expressed in CFU/g of sample.

- To determine ***E. coli*** β-glucuronidase, 1 mL of the serial decimal dilutions was seeded in petri dishes containing chromID Coli sterile medium (COLI ID-F, bioMérieux), plates were incubated at 44 ± 2 °C for 18-24 h. The counting was performed on plates in which growth occurred between 0 and 150 CFU and the results expressed as CFU/g of sample.
- **Yeasts and moulds** were determined by seeding 1 mL of serial decimal dilutions of RGP on the surface of Sabouraud Chloramphenicol Agar (Scharlab) plates and incubating for 5 days at 25 °C. The counting was performed on plates in which growth occurred between 0 and 150 CFU and the results expressed as CFU/g of sample.

For the detection of ***Salmonella*** **spp,** 25 grams of RGP sample were homogenized in aseptic conditions with 225 ml of buffered peptone water using Stomacher® 400 blender and incubated at 37 °C for 18 ± 2 h. After that, the sample was transferred into RVS selective enrichment Rappaport-Vasiliadis-Soja broth (bioMérieux) and Müller-Kaufmann broth (bioMérieux) and incubated at 41.5°C and 37°C for 24 h, respectively. Bacteria growths in these media were isolated on Xylose-lysine-deoxycholate (XLD) agar (Cultimed) and chromID *Salmonella* agar petri dishes (bioMerieux) by incubating at 37 °C for 24 h. The growth colonies were further identified by Api20 E analysis (bioMérieux) according to the manufacturer's instructions and confirmed by serological agglutination test (*Salmonella* Latex Test) according to the manual provided by the manufacturer on the test. The results were expressed as absence/presence of *Salmonella* spp. 25 grams of sample.

4.3.2.2. Chemical stability of the grape by-product during storage: antioxidant capacity

The antioxidant capacity of the GPE during the stability study was determined employing three complementary methods: the oxygen radical absorbance capacity (ORAC), the ABTS radical cation assay (ABTS), and the ferric reducing ability of plasma (FRAP) assays. GPE and its gastrointestinal *in vitro* digested fractions were prepared as indicated in sections 4.2.1 and 4.3.2, respectively.

ORAC$_{FL}$ assay

The ORAC assay was carried out following the protocol described by Huang et al. (2002) using fluorescein as a fluorescent probe. In this method the peroxyl radicals react with fluorescein resulting in the loss of fluorescence, which is detected with a fluorimeter. The inhibition of the oxidation produced by the peroxyl radicals determines the antioxidant capacity of the studied compound (Figure 10).

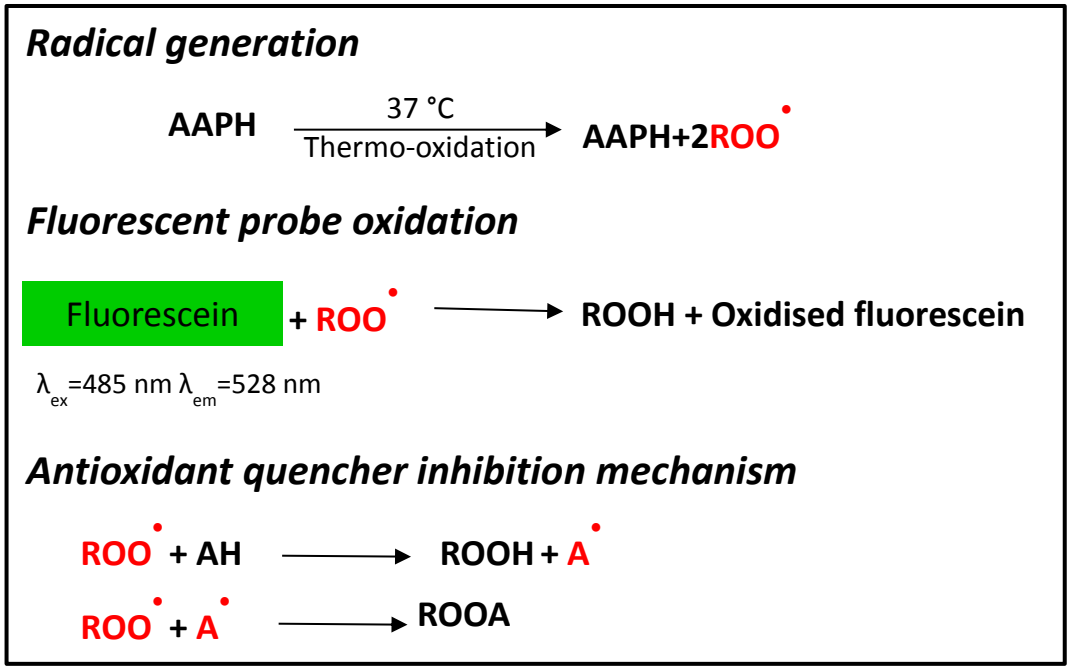


Figure 10. Principle of ORAC$_{FL}$ assay (Shahidi and Zhong, 2007). AAPH: 2,2'-Azobis(2-amidinopropane dihydrochloride, ROO: peroxyl radical, AH: antioxidant, R: alkyl group.

As the ORAC assay is extremely sensitive, the samples must be diluted appropriately before analysis to avoid interference. In this case, the GPE samples were diluted 1/1,000 (v/v). Twenty five microliters of samples and 150 µL of fluorescein (85 nM fluorescein in 75 mM phosphate buffer, pH 7.4, final concentration) were placed in a black 96-multiwell plate and preincubated at 37°C for 10 min in a plate reader (Bio Tek Microplate Reader, Winooski, VT USA). The oxidation reaction was initiated when the auto-injector added 350 µL of AAPH solution (153 mM in 75 mM phosphate buffer, pH 7.4) per well. The fluorescence readings were recorded at 485 nm and 528 nm excitation and emission wavelengths, respectively, every minute for 104 min. A calibration curve

of Trolox in concentrations of 6.25-50 μM (final concentration) in 75 mM phosphate buffer pH 7.4 were constructed and employed for quantification. An oxidation (AAPH, fluorescein and phosphate buffer) and non-oxidation (fluorescein and phosphate buffer) controls were also analysed in each plate.

$ORAC_{FL}$ value calculation was performed employing BioTek software and Excel Program. Normalized antioxidant curves (fluorescence versus time) were obtained, and the area under the fluorescence decay curve (AUC) was calculated employing equation 2:

Equation [2]: $AUC = 1+f_{104}/f_0+f_{103}/f_0+\ldots+f_0/f_0$

where f_0 is the initial fluorescence reading at 0 min, and f_i is the fluorescence reading at time i. The net AUC of each sample was calculated by subtracting the blank AUC to the sample AUC. Regression equations between net AUC and Trolox concentration were obtained and employed for calculation of the $ORAC_{FL}$ values expressed as μM Trolox equivalents (TE)/g dry matter.

TEAC or ABTS assay

The ABTS radical cation (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay was conducted according to Re et al. (1999). This method measures the ability of the antioxidants to scavenge a stable radical cation $ABTS^{\bullet+}$ (Figure 11). The radical $ABTS^{\bullet+}$ is intensively coloured and shows maximum absorption at 734 nm. When an antioxidant is present in the solution, the $ABTS^{\bullet+}$ radical can be reduced by antioxidants to the colourless product $ABTS^{2-}$. The percentage change of absorbance is recorded and results are expressed as TE.

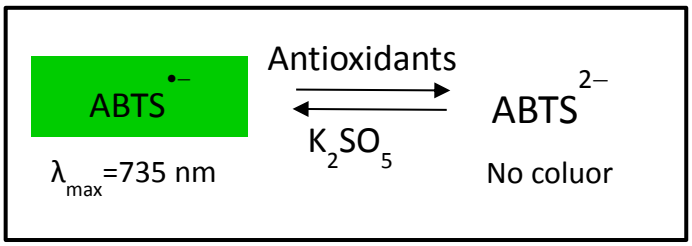


Figure 11. Principle of ABTS method (Shahidi and Zhong, 2007)

The radical $ABTS^{\bullet+}$ is generated by the reaction produced when $K_2S_2O_8$ (2.45 mM) and ABTS (7 mM) are incubated in the darkness at room temperature for 12-16 h. This solution was diluted 1/75 (v/v) with 5 mM phosphate buffer (pH 7.4) to obtain a working solution with an absorbance of 0.70 ± 0.02 at λ 730 nm. Ten microliters of GPE (1/2 dilution) were added with 3 mL of ABTS working solution and the absorbance at 734 nm was measured every 20 seconds during 7 min in a Beckman DU-600 spectrophotometer. A Trolox (1-5 mM) calibration curve was used for quantification. Samples were determined by quadruplicate and results are expressed as the mean ± SD as mM TE/g dry matter.

FRAP assay

The FRAP assay was carried out following the protocol described by Benzie and Strain (1996) with some modifications (Pulido et al., 2000). This method measures the reduction of the ferric tripyridyltriazine (TPTZ) complex to an intensely blue coloured ferrous complex by antioxidants in acidic medium. Antioxidant capacity is determined as an increase of absorbance at 593 nm (Figure 12).

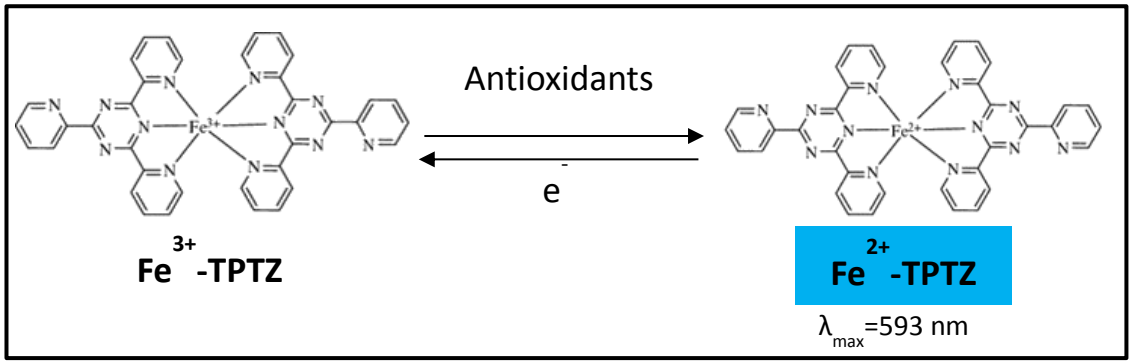


Figure 12. Principle of FRAP assay (Gülçin, 2011).

The FRAP working solution was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) dissolved in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$ solution. The reaction was carried out by mixing 30 µL of GPE (1/10 dilution) with 900 µL of FRAP working solution and 90 µL of distilled water, maintaining in the darkness for 30 min. Absorbance at 595 m was monitored every 20 seconds for 32 min using a Beckman DU-600 spectrophotometer. A standard calibration curve of Trolox was

constructed in a range of concentrations from 50 to 750 μM Trolox. Samples were determined by quadruplicate and results are expressed as μM TE/g DM.

4.3.3. Stability of the grape by-product to *in vitro* gastrointestinal digestion

The stability of the phenolic compounds present in RGP was also evaluated following *in vitro* gastrointestinal digestion. RGP was digested as previously described by Pereira-Caro et al. (2012) with some modifications. Pepsin from pig gastric mucosa (3.8 units/mg protein) and pancreatin from porcine pancreas (1.6 x 10^{-3} US Pharmacopeia (USP) specifications) were employed in the simulated gastric and intestinal digestion, respectively. The RGP sample (1 g) was diluted 1:8 (p/v) with 0.03 M HCl and the pH adjusted to 2.0 using 1M HCl and mixed vigorously by stirring at 3000 g at 37°C for 5 min.

Simulated gastric digestion: 1 mL of pepsin solution (40 mg/mL in 0.03 M HCl) was added to the RGP sample. The flasks were placed in a shaking water bath and the digestion was carried out at 3000 g, 37°C for 120 min. At the end of the gastric digestion, aliquots were taken for further analysis.

Simulated intestinal digestion: the sample resulting from the pepsin digestion was neutralized with 1 M NaOH adjusting the pH to 6.9 and then mixed with 2 mL of pancreatin solution (4 mg/mL in Britton Robinson buffer: 12.5 mM phosphoric acid, 12.5 mM acetic acid and 12.5 mM boric acid, pH 2.25) and bile salts (12 mg/mL in Britton Robinson buffer). The pH was adjusted to 7.5 with 6 M NaOH, and the mixture was agitated at 37°C for 4 h. After the pancreatic digestion, the digested samples were rapidly acidified (pH 2.0) to inhibit enzyme activity, aliquoted and stored at -20°C for further analysis.

All the aliquots (from both gastric and gastrointestinal digestions) were centrifuged at 4ºC, 2700 *g* or 10 min prior to phenolic compounds composition and antioxidant capacity analyses. Sample digestion controls without added digestive enzymes and bile salts were also run in parallel to differentiate the effects caused by the chemical environment in the assays. The antioxidant activity was determined by FRAP, ABTS and ORAC assays as described above. In all fractions, the phenolic composition was analysed by HPLC-DAD as indicated in section 4.3.1.2. after filtering samples through 45 μm PVDF filters.

4.4. Biological activity in cell models of oxidative stress and inflammation

The phenolic extracts **GPE, GCBE** and **YME** were evaporated by employing a rotary evaporator to eliminate methanol, acetone and HCl, and freeze dried. The powdered samples were homogenized with a mortar and pestle, dissolved in stock solutions of 100 mg/mL in 1% (v/v) DMSO and stored in aliquots at -20ºC for cell experiments.

Pure standards of gallic acid (GA), syringic acid (SA), chlorogenic acid (5-CQA), dihydroferulic acid (DHFA) and DHCA were dissolved 10 mM in 1% (v/v) DMSO in deionized water and diluted with serum free medium to prepare the different test solutions employed with cell cultures.

4.4.1. Cell culture and treatments

4.4.1.1. Caco-2 cell culture and treatments

For this study, human epithelial colorectal adenocarcinoma Caco-2 cells (Figure 13A) were employed as a model of intestinal cells to assess the protection against oxidative stress of GPE, GA and SA (Wang et al., 2016). Caco-2 cells were grown in DMEM F-12 medium supplemented with 10% FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin (Rodriguez-Ramiro et al., 2011). Caco-2 cells were incubated in 100 mm diameter culture dishes and maintained in a humidified incubator (Thermo Fisher, Madrid, Spain) in a 5% CO_2 and 95% air atmosphere at 37°C. The culture medium was changed every 2 days. Cells were usually split 1:3 when they reached 90% confluence. The day before the experiments, cells were changed to FBS-free medium to avoid potential FBS interference with the assays. All procedures with cells were carried out in aseptic conditions using laminar flow cabinets (Telstar AV 30/70, Madrid, Spain).

For Caco-2 cell treatment experiments, GPE stock solution of 10 mg/mL in 10% DMSO was prepared, aliquoted and stored at -20°C. The GPE stock solution was diluted the day of the experiment with FBS-free DMEM-F12 medium to prepare 0.1, 1 and 10 µg/mL solutions (final concentration of DMSO in the cell culture was 0.1%). GA and SA stock solutions (10 mM) were dissolved in 10%

(v/v) DMSO and diluted with FBS-free medium to prepare fresh working solutions the day of the experiment at concentrations of 0.1, 1 and 10 µM (final concentration of DMSO in the cell culture was 0.1%).

Two sets of experiments were designed to evaluate the antioxidant capacity of GPE on Caco-2 cells: i) direct effect experiments in which cells were incubated with different concentrations of GPE (0.1, 1 and 10 µg/mL in 0.1% DMSO), GA and SA (0.1, 1 and 10 µM in 0.1% DMSO) for 20 h; and ii) protection effect experiments, in which cells were pre-treated with the same concentrations of the GPE, GA or SA for 20 h before inducing an oxidative stress with FBS-free medium containing 400 µM *t*-BOOH for 3 h except in the ROS assay (see determination of ROS production). *t*-BOOH is an oxidative chemical able to produce cytotoxicity and oxidative stress on cells (Alia et al., 2005). After direct and protection experiments, Caco-2 cells were processed as detailed below for each assay.

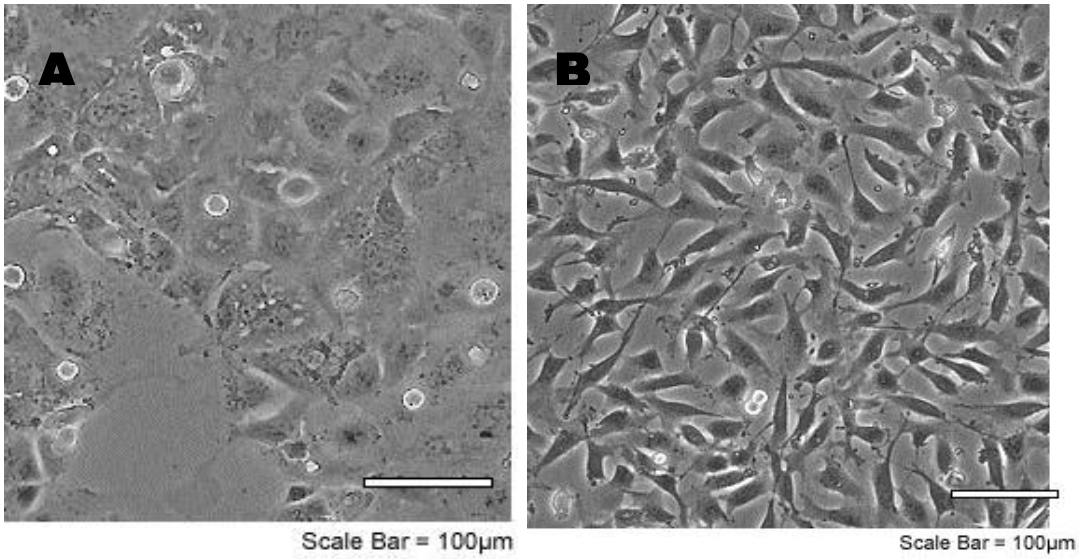


Figure 13. Confocal microscopy images of human epithelial colorectal adenocarcinoma Caco-2 (A) and human endothelium EA.hy926 (B) cells. Source: American Cell Type Collection.

4.4.1.2. EA.hy926 cells cell culture and treatment

For this study, human vascular endothelium EA.hy926 cells (Figure 13B) were employed as a model of endothelial cells to study the protective effect of GCBE, YM, 5-CQA, DHCA and DHFA against inflammation induced by tumour

necrosis factor alpha (TNF-α). EA.hy926 cells were grown in DMEM medium (4.5 g/L glucose) supplemented with 10% FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. EA.hy926 cells were maintained in 100 mm culture dishes in a humidified incubator containing 5% CO_2 and 95% air at 37°C. The culture medium was changed every other day in order to remove the not adherent and dead cells, and the cells were usually split 1:3 when they reached confluence. The day before the experiments, cells were changed to 2% FBS medium to reduce potential FBS interference with the assays not altering cell growth (Hou et al, 2011).

For EA.hy926 cells treatments, YME and GCBE stock solutions of 100 mg/mL in 10% (v/v) DMSO were prepared, aliquoted and stored at -20°C. The YME and GCBE stock solution were diluted the day of the experiment with FBS-free DMEM medium to prepare (1, 20 and 50 µg/mL in 0.1% DMSO). 5-CQA, DHCA and DHFA stock solutions (10 mM) were dissolved in 10% DMSO and diluted with FBS free medium to prepare fresh working solutions the day of the experiment at concentrations of 0.1, 1, 10, 20 µM 5-CQA in 0.1% DMSO, or 0.1, 0.3, 1, 10 µM DHCA and DHFA in 0.1% DMSO.

As for the studies of the effect of GPE in Caco-2 cells, the potential effect on EA.hy926 endothelial cells of GCBE, YME and its main hydroxycinnamic acid (5-CQA) and metabolites (DHCA and DHFA) were studied in direct and protective experiments. For experiments testing the direct effect, cells were incubated with YME, GCBE (1, 20 and 50 µg/mL in 0.1% DMSO), 5-CQA (0.1, 1, 10, 20 µM in 0.1% DMSO), DHCA and DHFA (0.1, 0.3, 1, 10 µM in 0.1% DMSO) for 24 h (save for the determination of ROS levels). To study the protective effects of the tested compounds, EA.hy926 cells were stimulated with the pro—inflammatory cytokine TNF-α; after pre-treatment with the same concentrations of the YME, GCBE, 5-CQA, DHCA and DHFA for 20 h, the medium was discarded, the cells were washed with PBS and stimulated with 10 ng/mL TNF-α for 24 h.

4.4.2. Evaluation of cell viability

WST-1 assay: The colorimetric WST-1 reagent assay was used to test cell viability with different extracts and compounds following manufacturer's instructions. This method is based on the reduction by viable cells of WST-1 reagent by NAD-dependent dehydrogenase activity to its water-soluble coloured

form formazan (Figure 14). The amount of dye formed directly correlates to the number of viable cells. Cells were seeded (100 µL, $4x10^3$ cells/well) in 96-well plates. After 24 h of incubation, the medium was removed and cells were treated with 100 µL of the different concentrations of the extracts and pure compounds for 20 h. Then, 10 µL of WST-1 reagent were added into the wells and the plates were incubated for 1 h. The absorbance at 440 nm was measured using a Bio-Tek microplate reader. Background control was also prepared by adding culture medium or sample plus WST-1 reactive in the absence of cells. Results are expressed as percentage of cell viability referred to the absorbance measured in control cells.

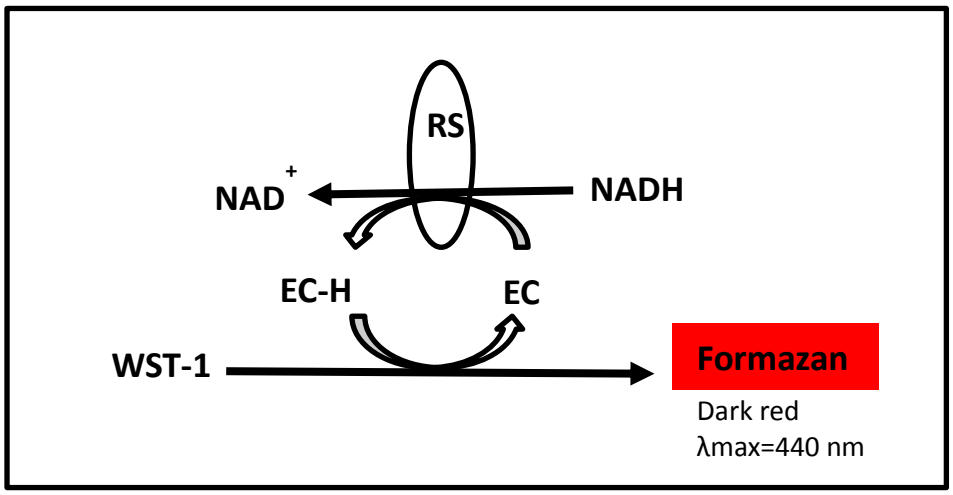


Figure 14. Principle of WST-1 assay. EC: electron coupling reagent, RS: mitochondrial succinate-tetrazolium-reductase system. Modified from Roche, 2011.

Crystal violet assay: Cell viability was determined by the crystal violet assay (Baeza et al., 2014). This method is based on the use of crystal violet, which is an acidophilic stain that accumulates in the cell nucleus and it correlates with the nuclear DNA content, and thus with viable cell number. Cells were seeded (10^4 cells/well) in 96-well plates, after 24 h incubation, cells were treated with the different compounds for 24 h, washed with 200 µL PBS and incubated with 50 µL of 0.2% (w/v) crystal violet solution (in 2%, v/v, ethanol) for 20 min. Wells were washed twice with 200 µL sterile Milli-Q water to remove excess of staining, and then 100 µL of 1% sodium dodecyl sulphate (SDS) were added to break down the cells and release the dye to estimate cell viability. The absorbance at 560 nm was measured using a microplate reader (Bio-Tek,

Winooski, VT, USA). Results are expressed as percentage of cell viability referred to the absorbance measured in control cells.

4.4.3. Evaluation of markers of oxidative stress

4.4.3.1. Cell cytotoxicity (LDH)

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay (Welder and Acosta, 1994). LDH is an intracellular enzyme and its release to the culture medium indicates cell damage. Cells (1.5×10^6 cells per plate) were seeded in 60 mm diameter plates and treated with 3 mL of the different concentrations of extracts and compounds as indicated in section 4.4.1. After treatment, the culture medium was collected; separately, the cells were scraped in 3 mL of PBS, sonicated to ensure the breakdown of the cell membrane and the release of the total amount of LDH, and centrifuged at 420 *g* at 4°C for 10 min. To determine LDH levels, 200 µL of 1.35 M Tris, 0.08 M pyruvate and 2 mg/mL NADH were added to 40 µL of culture medium or supernatant cell content in a 96 well plate and the absorbance at 340 nm was immediately read in a microplate reader for 5 min. The percentage of LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

4.4.3.2. Determination of ROS production

Cellular ROS were quantified by the dichlorofluorescein (DCF) assay using a microplate reader to screen the antioxidant effect of the different concentrations of each of the compounds studied (Alia et al., 2006). This assay is based on the measurement of the fluorescence produced by oxidation of the fluorescent probe DCFH-DH due to reactive oxygen species (Figure 15. Briefly, cells were seeded in 24-well plates (2×10^5 cells per well) in medium containing FBS and replaced with the FBS-free medium the following day. To evaluate direct effect of the studied compounds, 20 h after seeding the cells 10 µL of DCFH-DA (10 µM in DMSO) were added to the wells and cells were incubated at 37°C for 30 min in order that DCFH-DA could penetrate into the cells. Then, cells were washed with PBS (0.5 mL) and placed in fresh FBS-free medium with the different concentrations of extracts and compounds. ROS production was

monitored for 60 min by measuring the fluorescence in a microplate reader at 485 and 530 nm excitation and emission wavelengths, respectively.

To evaluate the protection effect of the studied compounds, cells were seeded (2 $\times 10^5$ cells per well) and left overnight before treating them with different extracts and compounds in FBS-free medium for further 20 h. Then, 10 µL of DCFH-DA (10 µM in DMSO) were added to the wells and cells were incubated at 37°C for 30 min. Cells were washed with PBS (0.5 mL) and FBS-free medium with 400 µM *t*-BOOH (Caco-2 cells) or 10 ng/mL TNF-α (EA.hy926 cells) were added to every well but controls with further incubation for 1 h. Control cells without *t*-BOOH or TNF-α treatment were used as a negative control. Control cells not treated with extracts or pure compounds but incubated with *t*-BOOH or TNF-α were used as a positive control. Fluorescent was read in a microplate reader at 485 and 528 nm excitation and emission wavelengths, respectively. Results are expressed as percent of fluorescence units from negative control.

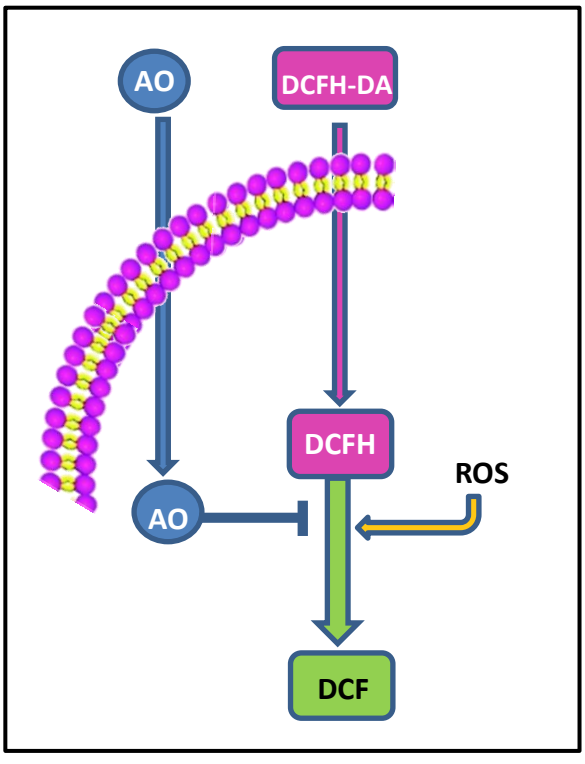


Figure 15. Diagram of the ROS assay. DCFH-DA penetrates the membrane of the cells and it is decomposed by intracellular esterase to DCFH, which is oxidized by the action of reactive oxygen species (ROS) to DCF (fluorescent compound). The presence of an antioxidant (AO) is able to delay the oxidation of DCFH.

4.4.3.3. Determination of GSH content

Glutathione (GSH) is a tripeptide and one of the major endogenous antioxidant defences of cells. GSH content was quantified by the fluorimetric assay developed by Hissin and Hilf (1976), with modifications. The method takes advantage of the reaction of reduced glutathione (GSH) with OPT at pH 8.0 to produce a highly fluorescent compound, which is measured at 340 and 460 nm excitation and emission wavelengths, respectively. Cells were plated in 60 mm diameter plates at a concentration of 1.5 x 10^6 cells/plate. Cells were treated with the different concentrations of extracts and compounds for 20 h, collected by scraping in 1.5 mL of PBS and centrifuged (420 g, 4°C, 5 min). Cellular lysates were obtained by adding 110 µL of 5% (w/v) trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells at 5500 gand 4°C for 30 min, 50 µL of the clear supernatant were transferred to wells in a 96-well plate. Then, 15 µL of 1 M NaOH was added to neutralize the sample and 175 µL of 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and finally 10 µL per well of a stock solution of OPT (10 mg/mL) in methanol were added. After 15 min at room temperature in the dark, fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the treatments were referred to those of a standard calibration curve of GSH. Protein concentration was measured with the Bio-Rad protein assay reagent following manual's instructions using *γ*-globulin as a protein standard.

4.4.3.4. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

GPx and GR are involved in the GSH turnover (Figure 16) being enzymatic antioxidant cell defenses. GPx and GR activities were determined following the protocols described by Alía et al. (2005). Briefly, cells were seeded in 100 mm diameter plates (2x10^6 cells/plate) and treated with the different extracts and standards for 20 h. Then cells were scraped and collected in 1.5 mL of PBS and centrifuged at 420 g, 4°C for 10 min. Cell pellets were resuspended and sonicated in 200 µL of 50 mM Tris containing 5 mM EDTA and 0.5 mM *β*-mercaptoethanol. Cell lysates were obtained by centrifuging at 1200 *g*, 4°C for 15 min.

Determination of GPx activity was based on the oxidation of GSH by GPx, using *t*-BOOH or 10 ng/ml TNF-α as a substrate, coupled to the disappearance rate of NADPH by GR. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione. Protein concentrations in the samples was measured using the Bio-Rad protein assay reagent following provider's instructions.

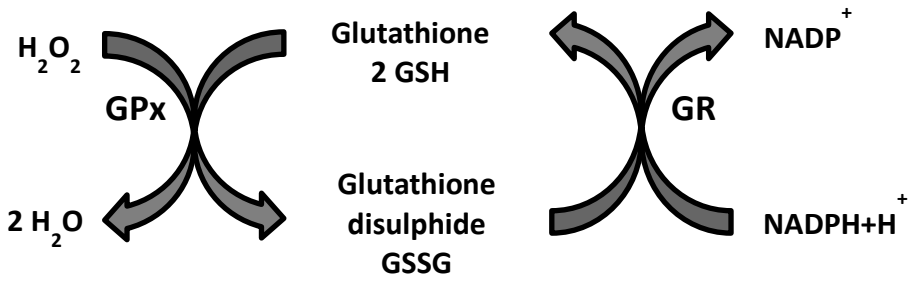


Figure 16. Scheme of GSH turnover by glutathione reductase (GR) and glutathione peroxidase (GPX).

4.4.3.5. Determination of protein damage

Carbonyl groups were determined as a biomarker of protein damage in Caco-2 cells following the protocol described by Levine et al. (1990) and modified by Martín et al. (2014). Cells were seeded in 100 mm diameter plates (2×10^6 cells/plate) and subjected to the different treatments. After treatment, cells were scrapped and collected in PBS. Cell pellets were obtained by centrifuging at 420 *g,* 4°C, 10 min, resuspended and sonicated in 110 µL of 0.25 M Tris buffer pH 7.4 containing 0.2 M sucrose and 5 mM DTT. The cytoplasmic content obtained by centrifugation was incubated with 4 volumes of 10 mM DNPH, and another aliquot treated with the same volume were incubated in 2 M HCl as control. All the tubes were kept in the darkness for 1 h at room temperature, mixing several times during incubation. The protein content was precipitated with the same volume of 10% trichloroacetic acid. After centrifuging and washing twice with 1 mL ethyl acetate: ethanol (1:1, v/v), the pellet was resuspended in 500 µL of 6 M guanidine and the absorbance was measured at 360 nm. Results are expressed

as nmol carbonyl per mg protein. Total protein concentrations was determined using the Bio-Rad protein assay reagent

4.4.4. Anti-inflammatory effects in EA.hy926 cells. Analysis of human endothelial Nitric Oxide Synthase (eNOS) levels

EA.hy926 cells were plated in 60 mm diameter plates at a density of 1.5 x 10^6 cells/plate. For direct effects, cells were incubated with YME, GCBE (1, 20 and 50 µg/mL in 0.1% DMSO), 5-CQA (0.1, 1, 10, 20 µM in 0.1% DMSO), DHCA and DHFA (0.1, 0.3, 1, 10 µM in 0.1% DMSO) for 24 h. To study the protective effects against TNF-α, cells were pre-treated with the same above concentrations of YME, GCBE, 5-CQA, DHCA and DHFA for 20 h, then the medium was discarded, cells were washed with PBS and stimulated with 10 ng/ml TNF-α for 24 h. Then, cells were collected by scraping in 1.5 mL of PBS and centrifuged (420 g, 4°C, 5 min). Then cells were resuspended in PBS, sonicated and centrifuged (15300 g, 4°C, 30 min). The supernatants were collected, and protein concentration was determined using the Bio-Rad protein assay reagent. Cell lysates were aliquoted and stored at -80 ºC until eNOS measurement.

Concentration of eNOS in cell lysates was determined using a human eNOS ELISA kit (BD Biosciences) based on sandwich ELISA following manufacturer's instructions. Briefly, 96 well plates were pre-coated with 100 µL of mouse anti-human eNOS capture antibody (1 µg/mL) overnight at room temperature. Wells were washed (3 times) with Wash Buffer to eliminate non-binding antibody, blocked with 300 µL of Blocking Solution at room temperature for 1 h and washed again with Wash Buffer. One hundred microliters of cell lysates were added per well and the plate was incubated at room temperature for 2 h. After that, 100 µL/well of detection antibody were added and incubated at room temperature for 2 h. The plate was washed three times with Wash Buffer to remove the excess of secondary antibody. Quantification of eNOS was performed by adding 100 µL of Streptavidin-HRP working dilution to each well and incubating in darkness at room temperature for 20 min. The colorimetric reaction was stopped by adding 50 µL of Stop Solution to each well and the absorbance was measured at 450 nm with a plate reader. A calibration curve was also run in every plate using recombinant human eNOS standard in a range of

concentration from 78.1 to 5000 pg/mL. Results were expressed as pg eNOS/mg protein.

4.5. Antiproliferative effects of green coffee and yerba mate extracts and phenolic compounds on human cancer cell lines

Human oesophageal (OE-33), lung (A549), colon (Caco-2) and urinary bladder (T24) carcinoma cells were employed as models of cancer cells. CCD-18Co non-carcinogenic cells was also used as a model of healthy cells to study the pro/antiproliferative effect of GCBE, YME, their main phenolic compounds [5-CQA, 3,5-di-CQA, caffeic acid (CA), ferulic acid (FA)], caffeine, and metabolites (DHCA, DHFA).

CCD-18Co cell line (passages 18-25) was incubated in MEM supplemented with 10% FBS, sodium bicarbonate (1.5 g/L), 1 mM sodium piruvate, 0.1 mM NEAA and 50 mg/L of antibiotics (gentamicin, penicillin and streptomycin). A-549 and Caco-2 cells were incubated in DMEM F-12 medium supplemented with 10% FBS and 50 mg/L of antibiotics (gentamicin, penicillin and streptomycin). T24 cell line was grown in McCoy's 5A medium supplemented with 10% FBS and 50 mg/L of antibiotics (gentamicin, penicillin and streptomycin). OE 33 cells were incubated in RPMI 1640 with glutamine medium supplemented with 10% FBS and 50 mg/L of antibiotics (gentamicin, penicillin and streptomycin). Cells were grown in culture dishes (100 mm diameter) in a humidified atmosphere of 5% CO_2 and 95% air at 37ºC.

In the viability and proliferation assays, the tested concentrations ranged from 10 to 1000 µg/mL for GCBE and YME, and from 10 to 1000 µM for the pure standards, in order to study the effect of physiological and supra-physiological doses, simulating a situation of normal or overconsumption, respectively. The exposure to the studied compounds and GCBE and YME during 2 and 24 h was established to mimic acute and chronic consumption, respectively.

Further studies of cytotoxicity (LDH leackage) and reactive oxygen species (ROS) production were also carried out in the non-cancer cells with lower doses of 5-CQA, 3,5-DCQA, DHCA, CA (0.1-10 µM) and for all the studied cells

for YME (0.1-10 µg/mL). Control cells were treated with 0.1% of DMSO in comparison with treated cells. The day before the experiments, cells were changed to FBS-free medium to avoid potential FBS interference with the assays.

4.5.1. Cell viability and cell proliferation tests (WST-1 assay)

To determine the effect of the tested extracts and pure compounds on cell viability, the WST-1 assay was carried out as described above. Cells were treated with 100 µL of the different concentrations of pure standards or extracts for 2 or 24 h. The absorbance was measured at 440 nm using a microplate reader (Bio-Tek, Madrid, Spain).

BrdU colorimetric assay was used to study cell proliferation. This method is based on the incorporation of the pyridine analogue 5-bromo-2'-deoxyuridice (BrdU) instead of thymidine into the DNA of proliferating cells and its quantification by direct ELISA with anti-BrdU antibodies. Cells were seeded at 10^4 cells per well (100 µL) in 96-well microplates, incubated for 24 h for attachment, and the different compounds were added for 2 or 24 h. After the incubation period, cells were labelled with BrdU for 4 h. Cells were fixed with Fixation Solution (200 µL/well) at room temperature for 30 min, and the anti-BrdU-POD antibody was added and incubated at room temperature for 90 min. Cells were washed with Wash Solution (300 µL/well) four times and incubated with 100 µL of TMB Solution. The absorbance was measured at 370 nm. Results are expressed as a percentage of cell proliferation referred to the absorbance determined in untreated control cells in the same plates.

4.6. Statistical analysis

SPSS version 19.0 program for Windows (SPSS Inc., Chicago, Ill., U.S.A.) was used for the statistical analysis of data. Homogeneity of variances was checked by the test of Levene. Multiple comparisons within the different treatments were carried out using One-way ANOVA followed by Bonferroni test when variances were homogeneous or by the Games-Howell test when variances were not homogeneous. Differences were considered as statistically significant when $p < 0.05$.

Results and Discussion

Chapter 1

This chapter attempts to better understand the potential of a red grape pomace as a functional ingredient or nutraceutical. Firstly, the effects of storage and *in vitro* digestion on the phenolic content and antioxidant capacity of a red grape pomace were evaluated. Subsequently, the protective effects of the phenolic extract from the grape by-product and its main hydroxybenzoic acids were studied in Caco-2 cells under an oxidative stress condition.

Study 1 was published as: Wang, S., Amigo-Benavent, M., Mateos, R., Bravo, L., Sarriá, B., (in press). Effects of *in vitro* digestion and storage on the phenolic content and antioxidant capacity of a red grape pomace. International Journal of Food Sciences and Nutrition. DOI: 10.1080/09637486.2016.1228099

Study 2 was published as: Wang, S., Mateos, R., Goya, L., Amigo-Benavent, M., Sarriá, B., Bravo, L., 2016. A phenolic extract from grape by-products and its main hydroxybenzoic acids protect Caco-2 cells against pro-oxidant induced toxicity. Food and Chemical Toxicology 88, 65-74.

Study 1

Effects of *in vitro* digestion and storage on the phenolic content and antioxidant capacity of a red grape pomace.

Summary

Red grape pomace (RGP) is a major winery by-product with interesting applications due to its high phenolic content and antioxidant capacity. Effects of *in vitro* gastrointestinal digestion and storage on the phenolic content and antioxidant capacity of RGP were studied. RGP polyphenols were stable under stomach- mimicking conditions and more sensitive to small intestine conditions, drastically reducing anthocyanins and flavonols. After 3 and 6 month storage, at either 4 or 25ºC, there were no changes in the total phenolic and condensed tannin content, or antioxidant capacity (evaluated by ABTS, FRAP, ORAC assays); however, after 9 months these parameters decreased. Contrarily, chromatic b* values were higher, thus the samples had more intense red colour, which may be related to the increased condensed tannin content. Storage time or temperature induced no changes in microbiological load. RGP preserves high antioxidant capacity after storage and *in vitro* digestion and thus presents potential as a functional ingredient or nutraceutical.

Keywords: antioxidant, phenolic compounds, grape by-product, storage, *in vitro* digestion

Introduction

Grape is one of the largest fruit crops worldwide. Every year the processing of grapes for wine and juice yields an estimated global amount of at least 10 million tons of by-products (Kammerer et al., 2004). Grape pomace is a complex mixture, mainly consisting in peels (skin), seeds and stems. Grapes are appreciated for their rich content of phenolic compounds like gallic acid, ellagic acid and resveratrol, and a wide variety of procyanidins. Grape seeds are rich in extractable phenolic antioxidants such as phenolic acids, flavonoids, procyanidins and resveratrol, while red grape skin contains abundant anthocyanins (Yu et al., 2013). Numerous studies support that grape phenolic compounds present antioxidant activity among other biological activities inducing beneficial health effects and disease prevention in humans (Bagchi et al., 2000; Xia et al., 2010). Grape phenolic extracts have shown to modulate vascular cell function, inhibit oxidation of human low-density lipoproteins (Bradamante et al., 2004), inhibit the proliferation of cancer cells (Chung et al., 2012), modulate the expression of antioxidant enzyme systems (Wang et al., 2016), and protect against free radical-mediated tissue injury (Xia et al., 2010), among other biological activities. In addition to phenolic compounds, grape residues are an important source of dietary fiber (Saura-Calixto, 2011). For all the aforementioned reasons, grape by-products present great potential as a functional ingredient and have become very popular in the recent years, with many nutraceuticals and dietary supplements containing grape by-products.

Storage conditions have shown to have a major impact on the properties of phenolic compounds in red wine grapes (Arapitsas et al., 2016). Regarding storage temperature, in a recent experiment carried out in wine waste encapsulated extracts, slower degradation rates and longer stability was described at 4ºC than at room temperature (Aizpurua-Olaizola et al., 2016). Storage time also affects the stability of bioactive compounds in grape pomace, which significantly decreased after 16 week storage at 15 ± 2°C (Tseng and Zhao 2012). Other authors observed that storage influenced flavonol contents differently depending on the berry type, so that quercetin content decreased markedly (40%) in bilberries and lingonberries, but not in black currants or red raspberries during 9 months of storage at -20°C (Häkkinen et al., 2000). According to a study on the influence of storage conditions on the stability of anthocyanins in grape skins, decomposition rate significantly depends on the

time and temperature of storage, with the effect of storage time being the most important whereas the presence or absence of light exert a negligible impact (Moraisa et al., 2002).

Although the phenolic compounds may have a strong antioxidant activity *in vitro*, their bioactivity depends on the extent to which they are absorbed, distributed, metabolized and eliminated from the body, i.e. bioavailability determine biological activity. However, assessment of true bioavailability of any phytochemical in animals or humans is complex and expensive. *In vitro* gastrointestinal digestion is commonly used as an approach to gain information on the release of phenolic compounds from the food matrix and their stability under digestive conditions. The *in vitro* digestion procedure is a simple, rapid method that allows the screening of multiple samples and may provide data on the relative potential bioavailability (i.e. bioaccesibility) of different polyphenolic components from fresh, extracted, and processed foods (McDougall et al., 2005). Some authors have studied the effect of *in vitro* gastrointestinal digestion on the stability of pure phenolic compounds (Pereira-Caro et al., 2012b) and release of polyphenols from beverages like juice (Gil-Izquierdo et al., 2002), as well as from solid food matrix (Tagliazucchi et al., 2010) and plant extracts (Pinacho et al., 2015). However, to our knowledge, the *in vitro* gastro-intestinal digestion of a grape by-product, looking into the changes in the contents of its phenolic components along the digestion process, has not been previously studied.

The aim of this work was to encompass the effect of both storage time and temperature on the phenolic composition and antioxidant properties of a powdered red grape pomace, and assess the influence of *in vitro* gastrointestinal digestion on these parameters to obtain a good estimate of the bioactive potential of the product. The impact of the by-product's nutritional quality as well as its sustainability in the food chain were thus evaluated.

Results and discussion

Phenolic content and antioxidant capacity in basal, non-stored RGP samples

Grape composition is greatly influenced by different factors, including climate, irrigation, management practices, degree of ripeness, berry size and grapevine variety (Tesic et al., 2007). Higher concentration of phenolic compounds in seeds than in skin has been described for almost all the studied grape varieties (Negro and Micelli, 2003; Sandhu and Gu, 2010; Deng et al., 2011; Rockenbach et al., 2011a; Ćurko et al., 2014). The grape by-product studied here is a dry powder mixture of skin and seeds of Tempranillo grapes. The total phenolic content observed in the red grape pomace phenolic extract (RGPE, 4.84 ± 0.13 g gallic acid equivalents (GAE)/100 g dry matter (d.m.), Table 8) was higher than the values reported for other grape by-products. According to Cerda-Carrasco et al. (2015) the phenolic content was about 1.3, 1.4, 1.7, and 1.9 g GAE/100 g for varieties Camenere, Cabernet Sauvignon, Chardonnay and Sauvignon-Blanc, respectively, whereas Deng et al. (2011) reported 2.14 ± 0.43 g GAE/100 g of total polyphenols in Pinot Noir grape dried skin. However, other works showed similar results to those here presented; Negro and Miceli (2003) reported 3.33 ± 0.03 and 4.19 ± 0.04 g GAE/100 g d.m. in grape extracts obtained from skin and pomace, respectively. The total phenolic content of Syrah and Petit Verdot red pomaces, extracted using ethanol/water, was 3.9 and 6.6 g GAE/100 g, respectively (Melo et al., 2015) and the grape pomace mixture of 65% Bordeaux, 25% Isabel and 10% BRS Violet varieties showed a total phenolic content of 4.1 ± 0.1 g GAE/100 g, whereas the pomace of Cabernet Sauvignon, Merlot and Terci presented a content of approximately 2.7, 2.5 and 3 g GAE/100 g, respectively (Ribeiro et al., 2015). Nevertheless other research groups reported higher values, such as Monagas et al. (2005), describing 10.1 ± 0.9 to 15.9 ± 0.2 g GAE/100 g in commercial supplements derived from grape skin, and Antoniolli et al. (2015) who observed 19.6 g GAE/100 g in grape pomace of cv. Malbec. Apart from the factors aforementioned, differences in the phenolic contents described may be due to the extraction procedure applied. The importance of the extraction procedure is evidenced in Ky and Teissedre (2015), as a grape pomace skin extracted with water showed a total phenolic content of 14.6 g GAE/100 g whereas the extraction with ethanol/water (70:30) yielded 22.5 g GAE/100 g.

Table 8. Total polyphenol and condensed tannin content, antioxidant capacity, pH and moisture of red grape pomace at the start of the stability study.

Total polyphenols (g GAE /100 g d.m.)	4.84 ± 0.13
Condensed tannins (mg/ g d.m.)	59.8 ± 6.17
ABTS (µmol TE/g d.m.)	545.13 ± 27.40
FRAP (µmol TE/g d.m.)	402.47 ± 10.25
ORAC (µmol TE/g d.m.)	1921.36 ± 196.12
pH	2.37 ± 0.02
Moisture (%, w/w)	5.68 ± 0.19

Data express mean ± standard deviation (n = 4). d.m.: dry matter; GAE: gallic acid equivalents; TE: Trolox equivalents.

Characterization of the phenolic composition in the RGPE is shown in Table 9. The pomace was especially rich in phenolic acids (1.64 mg/g), accounting for up to 83% of the total polyphenols in RGP. Of these, gallic, ellagic and protocatechuic acids were the most abundant phenols. Quercetin, together with its glucoside and kaempferol-3-*O*-glucoside, were the major flavonols, with up to 0.30 mg/g DM of total flavonols in the grape pomace. Only minor amounts of anthocyanins were detected (0.026 mg/g), with malvidin-3-*O*-glucoside as the main anthocyanin. This is in agreement with many other varieties of red grape by-product extracts, which described malvidin-3-*O*-glucoside as the most abundant anthocyanin (Kammerer et al., 2004; Melo et al., 2015). However, a low content of total anthocyanins was quantified in RGPE. There are several reasons that may explain this observation. Anthocyanin values in grape pomace vary depending on the extraction, analytical methods and grape varieties (Thimothe et al., 2007). In the present study, we used the same chromatographic method to identify phenolic acids, flavonols, and anthocyanins. In other studies (Kammerer et al, 2004; Antoniolli et al, 2015; Melo et al, 2015; Ribeiro et al, 2015) a different chromatographic system was used to separate anthocyanins and non-anthocyanin phenolic compounds. It cannot be ruled out that if we had used a specific method for anthocyanins a higher concentration might have been observed. However, it should also be considered that anthocyanins are highly reactive species, easily degraded. Factors that affect the stability of anthocyanins include structure, pH, temperature, light, etc. (Rodriguez-Saona et al., 1999), storage conditions (Moraisa et al., 2002), processing temperature, as well as

other intrinsic properties such as light, oxygen, the presence of enzymes, proteins and metallic ions (Garcia-Viguera et al., 1999), etc. Anthocyanins may also be degraded by oxidative mechanisms involving the enzyme polyphenol oxidase in the presence of another substrate, such as gallic acid (Welch et al., 2008). Since the RGPE was obtained from a winery byproduct, an extensive extraction of anthocyanins during wine-making as well as the potential loss of residual anthocyanins from the grape pomace during ulterior treatment (drying, milling, etc.) might also account for the negligible content of anthocyanins in the RGPE.

Although the flavanols catechin and epicatechin have been reported in grape pomace (Yilmaz and Toledo 2004; Ribeiro et al., 2015), the absence of catechins and epicatechins in RGPE had also been previously observed when the characterization of the phenolic compounds in the extract of the same grape product prepared by the conventional organic solvent extraction (0.8% HCl in methanol/water, 50/50 v/v, and acetone/water, 70/30 v/v) and analysed by a highly sensitive analytical method (HPLC-ESI-QToF) (Wang et al., 2016). We are not aware of the conditions involved in the production and storage of the red grape pomace, which might have affected the final content of these flavonoids. It has been described that catechin and epicatechin contents are reduced during storage of grape seeds collected after wine-making procedures, where storage temperature and particularly relative humidity have an important impact (Hatzidimitriou et al., 2007).

Overall, total polyphenols quantified in the RGPE amounted to 1.967 mg/g d.m. (Table 9). The content of condensed tannins in the present work was 59.8 ± 6.17 mg/g DM, which is much higher than the results in skin (8.6 ± 0.8 mg/g d.m.) and proximate to that of seeds (64.1 ± 0.8 mg/g d.m.) previously described in red grape marc extracts (Negro and Miceli 2003). This suggests a richer content in seeds than in skin in RGP.

Table 9. Composition of phenolic compounds in red grape pomace initially and after *in vitro* digestion.

Peak	Compound	RT (min)	λmax	Molecular Ion	M.F.	Initial phenolic content	Gastric Digestion	Pancreatic Digestion
Phenolic acids (280 nm)						**μg/g d.m.**	**μg/g d.m.**	**μg/g d.m.**
1	Gallic acid	3.2	272	169.0142	$C_7H_6O_5$	530.78±3.42[a]	534.47±6.6[a]	511.78±6.03[b]
2	Protocatechuic acid	5.4	260, 292	153.0193	$C_7H_6O_4$	255.17±1.79[a]	258.14±4.80[a]	241.23±2.14[b]
3	Caftaric acid	6.2	296. 324	311.0409	$C_{13}H_{12}O_9$	91.05±3.07[a]	88.78±2.34[a]	87.39±1.71[a]
4	*p*-hydroxybenzoic acid	6.5	285	137.0244	$C_7H_6O_3$	49.99±2.60[a]	52.58±2.26[a]	48.96±0.49[a]
5	*Cis*-coutaric acid	8.6	294, 309	295.0459	$C_{13}H_{12}O_8$	8.52±1.04[a]	8.88±1.60[a]	7.12±0.57[a]
6	*Trans*-coutaric acid	9.9	295, 314	295.0459	$C_{13}H_{12}O_8$	32.24±1.28[a]	28.49±1.05[b]	15.36±1.36[c]
7	Fertaric acid	10.2	297, 331	325.0565	$C_{14}H_{14}O_9$	120.06±1.48[a]	115.74±5.67[a]	106.51±2.16[b]
8	Vanillic acid	10.8	260	167.0350	$C_8H_8O_4$	102.48±2.36[a]	96.54±1.83[b]	80.97±1.92[c]
9	Syringic acid	15.3	279	197.0455	$C_9H_{10}O_5$	112.75±2.18[a]	102.67±5.78[b]	93.98±3.52[b]
10	Ellagic acid	20.8	254, 368	300.9990	$C_{14}H_6O_8$	335.45±3.31[a]	333.17±4.58[a]	289.24±2.22[b]
Total phenolic acids						**1,638.49±22.52[a]**	**1,619.47±36.53[a]**	**1,482.53±22.12[b]**
Flavonols (360 nm)								
11	Rutin	19.5	256, 354	609.1461	$C_{27}H_{30}O_{16}$	5.70±0.71[a]	5.52±0.35[a]	4.70±0.32[a]
12	Quercetin-3-*O*-glucoside	20.8	256, 354	463.0882	$C_{21}H_{20}O_{12}$	38.33±0.41[a]	33.63±0.49[b]	28.94±0.56[c]
13	Quercetin-3-*O*-glucuronide	21.9	254, 353	477.0675	$C_{21}H_{18}O_{13}$	29.31±0.47[a]	24.55±0.75[b]	21.19±0.14[c]
14	Miricetin-3-*O*-glucoside	22.1	258, 358	479.0831	$C_{21}H_{20}O_{13}$	1.90±0.21[a]	1.74±0.23[a]	1.43±0.18[a]
15	Isoramnetin-3-*O*-glucoside	22.8	254, 354	477.1038	$C_{22}H_{22}O_{12}$	15.29±0.28[a]	16.47±0.72[a]	11.28±0.28[b]
16	Kampherol-3-*O*-glucoside	25.4	264, 348	447.0933	$C_{21}H_{20}O_{11}$	28.43±0.15[a]	25.50±1.29[b]	21.90±0.32[c]
17	Siringetin-3-*O*-glucoside	27.1	252, 360	507.1144	$C_{23}H_{24}O_{13}$	23.58±0.47[a]	24.61±0.64[a]	18.45±0.23[b]
18	Miricetin	28.2	254, 368	317.0303	$C_{15}H_{10}O_8$	25.27±0.43[a]	17.66±1.41[b]	8.34±0.58[c]
19	Quercetin	37.5	255, 370	301.0354	$C_{15}H_{10}O_7$	87.53±2.32[a]	72.46±2.40[b]	47.70±1.07[c]
20	Laricetrin	38.1	250, 372	331.0459	$C_{16}H_{12}O_8$	19.09±0.35[a]	17.89±0.23[b]	13.64±0.28[c]
21	Kaempherol	44.1	264, 363	285.0405	$C_{15}H_{10}O_6$	14.94±0.23[a]	15.46±0.23[a]	11.35±0.24[b]
22	Isoramnetin	44.7	256, 368	315.0510	$C_{16}H_{12}O_7$	13.11±0.15[a]	13.40±0.30[a]	10.97±0.12[b]
Total flavonols						**302.48±6.18[a]**	**268.90±9.02[b]**	**199.92±4.32[c]**

Anthocyanins (520 nm)								
23	Delphinidin-3-*O*-glucoside	10.7	520	465.1028	$C_{21}H_{20}O_{12}$	4.82±0.20[a]	1.55±0.09[b]	n.d.
24	Cyanidin-3-*O*-glucoside	14.2	510	449.1078	$C_{21}H_{20}O_{11}$	1.99±0.13[a]	1.15±0.09[b]	n.d.
25	Petunidin-3-*O*-glucoside	14.9	528	479.1184	$C_{22}H_{23}O_{12}$	4.55±0.67[a]	2.55±0.26[b]	n.d.
26	Peonidin-3-*O*-glucoside	18.4	520	463.1235	$C_{22}H_{23}O_{11}$	2.87±0.20[a]	2.90±0.31[a]	n.d.
27	Malvidin-3-*O*-glucoside	19.0	522	493.1341	$C_{23}H_{25}O_{12}$	12.33±0.8[a]	9.34±0.38[b]	n.d.
Total Anthocyanins						**26.55±2.01[a]**	**17.47±1.12[b]**	**n.d.**
Total Phenols						**1,967.52±30.71[a]**	**1,905.85±46.68[a]**	**1,682.45±26.44[b]**

Results are mean values ± standard deviation. DM: dry matter; MF: Molecular formula; n.d.: not detected; RT: retention time.

There are disparities in the assays used to evaluate antioxidant activity of extracts, as well as in the expression of results and use of reference antioxidants (Fontana et al., 2013). RGPE showed a high antioxidant capacity (Table 8). The reducing power of RGPE determined by the FRAP assay (402 ± 10 µmol TE/g d.m.) was similar to that described for Cabernet Sauvignon pomace seed (449.18 ± 15.08 µmol TE/g) although higher to that for the skin (170 ± 3 µmol TE/g; Xu et al., 2010) and the pomace of red grapes *V. vinifera* L. and *V. labrusca* L. (117-249 µmol TE/g) (Rockenbach et al., 2011b). Regarding $ABTS^{\bullet+}$ scavenging capacity, RGPE showed values (545 ± 27 µmol TE/g d.m.) slightly below the range described for red grape pomace of Petit Verdot and Syrah (626 and 653 µmol TE/g, respectively; Melo et al., 2015), although above those described for *V. vinifera* Cabernet Sauvignon pomace seed (449.2 ± 15.1 µmol TE/g d.m.) and pomace skin (258 ± 16 µmol TE/g; Xu et al., 2010). Lower $ABTS^{\bullet+}$ scavenging capacity has been reported in red grape pomaces, 193-485 µmol TE/g (Rockenbach et al., 2011b).

In few studies the ORAC method has been used to determine antioxidant capacity in extracts derived from grape pomace. Based on results from the ORAC assay, the total antioxidant capacity of RGPE was higher than values described by other authors for Muscadine (245.9 ± 6.8 µmol TE/g d.m.) and Chardonnay (450.5 ± 74.0 µmol TE/g d.m.) grape seed extracts (Yilmaz and Toledo 2004). ORAC values between 276.6 and 1538.4 µmol TE/g in fresh weight of grape seeds from Noble and Fry cultivars, respectively, have been reported (Sandhu and Gu 2010), with lower values in skin (26.0-77.5 µmol TE/g) and pulp (2.3-4.6 µmol TE/g). These differences in antioxidant capacity reflected the different phenolic content of the whole grape fractions, which on average 87.1% of the total phenolic content of the grape corresponded to the seed, 11.3% to the skin and 1.6%, to the pulp (Sandhu and Gu 2010). Supplements produced from grape skin and pomace showed ORAC values from 1380 to 21400 µmol TE/g (Monagas et al., 2005). However, other groups have described higher levels of ORAC, such as 2756 ± 109 µmol TE/g in Malbec grapes (Antoniolli et al., 2015). Furthermore, a freeze-dried grape phenolic extract obtained by enzymatic extraction showed an ORAC value of 4239 µmol TE/g (Rodríguez-Rodríguez et al., 2012).

Effects of storage temperature and time on RGP

The influence of both temperature and storage time on total phenolic and condensed tannin content, antioxidant capacity, moisture, colour and microbiological safety of the RGP was evaluated.

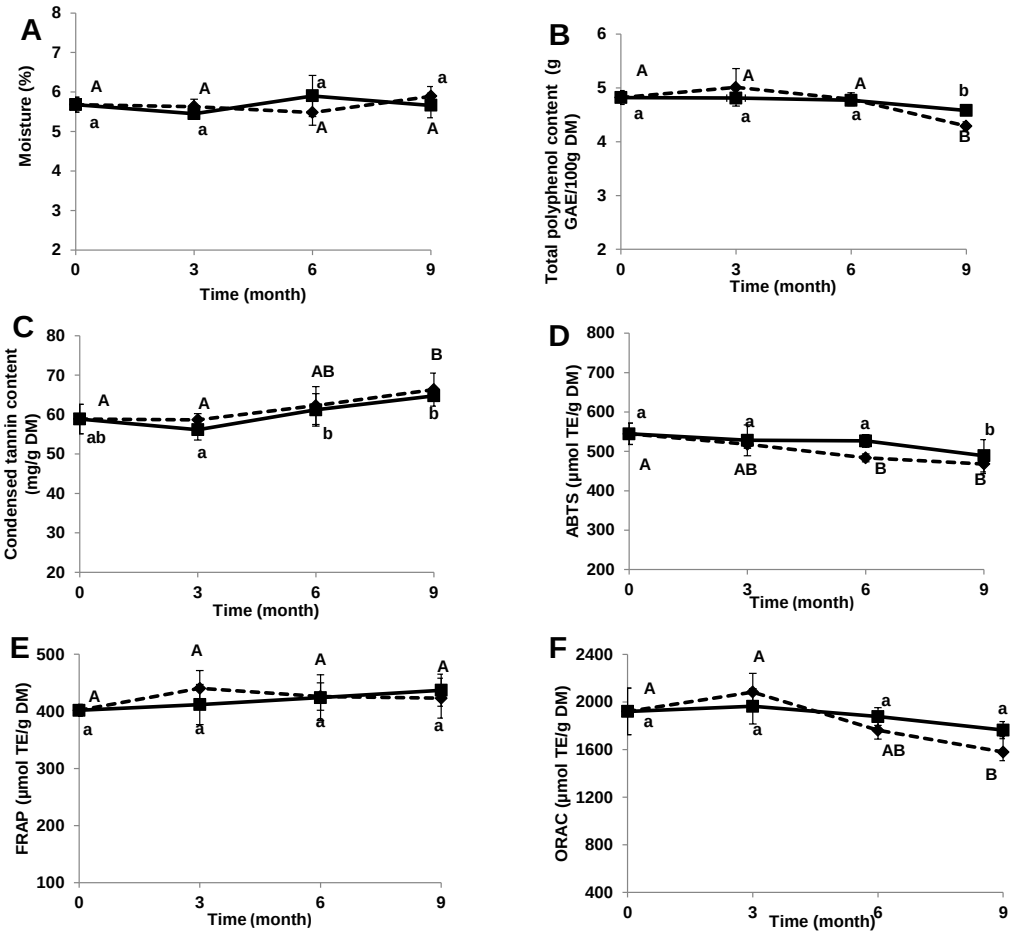


Figure 17. Changes in moisture, total polyphenol and condensed tannin contents and antioxidant capacity (determined by the ABTS, FRAP and ORAC assays) of the grape by-product according to storage time and temperature. The dashed and solid lines correspond to 4 and 25ºC, respectively. (A) Moisture. (B) Total polyphenol content. (C) Condensed tannin content. (D) $ABTS^{\bullet+}$ (E) FRAP. (F) ORAC. Data expressed as the mean ± SD (n= 4–8). Different letters in the figure indicate statistically significant differences due to time, within 4ºC (capital letters) and 25ºC (lower case letters) by the Bonferroni test ($p < 0.05$).

Effect of storage temperature and time on moisture and colour parameters

No significant changes in moisture were observed during the stability test (Figure 17a). Average colour values were obtained by measuring L*, a* and b* chromatic parameters (Table 10). The positive values for a* (from green to red) before the beginning of the storage experiment indicate that the colour of RGP was predominantly red, which is characteristic of Tempranillo grapes. During storage this parameter gradually increased, with samples having a more intense red colour towards the end of the experiment. In addition to the effect of time, there was a significant influence of temperature, whereby at lower temperature (4 ºC) higher a* values were observed at 3 and 6 month storage. Regarding the chromatic component b* (from blue to yellow), values decreased at month 3 but then recovered, not showing differences compared to the initial values after 9 months of storage. Lightness (L*) decreased along the storage compared to basal values. Colour changes during storage are likely to be due to polymerization reactions mediated by phenolic compounds (García-Alonso et al., 2003), which agrees with the increase in condensed tannins observed after 9 month storage (see next section). Similarly, Johnston and Morris (1997) have described a polymerization phenomenon mediated by phenolic compounds in Cabernet Sauvignon and Nobel wine.

Table 10. Chromatic components of the red grape pomace according to storage time and temperature. L* is the luminance or lightness, a* goes from green to red and b* from blue to yellow.

	0	3 months		6 months		9 months	
		4°C	25°C	4°C	25°C	4°C	25°C
L*	43.01 ± 0.4^{aA}	41.88 ± 0.32^{B}	41.55 ± 0.2^{b}	41.84 ± 0.25^{B}	41.26 ± 0.11^{b}	41.58 ± 0.11^{B}	41.82 ± 0.51^{b}
a*	7.37 ± 0.1^{acA}	7.33 ± 0.19^{A}	6.84 ± 0.25^{a}	7.99 ± 0.19^{B}	7.45 ± 0.28^{b}	9.38 ± 0.28^{C}	9.42 ± 0.13^{c}
b*	3.24 ± 0.08^{aA}	2.81 ± 0.18^{B}	2.69 ± 0.1^{b}	3.67 ± 0.2^{C}	3.28 ± 0.10^{a}	3.25 ± 0.10^{A}	3.47 ± 0.07^{a}

Data express mean ± standard deviation (n = 4).

Different letters indicate statistically significant differences due to time, within 4ºC (capital letters) and 25ºC (lower case letters) according to the Bonferroni test. Statistical significance was set at $p<0.05$.

Effect of storage temperature and time on the phenolic compounds and condensed tannin content and antioxidant capacity

According to our results, there were no significant differences in the total phenolic content from 0 to 6 months at any of the temperatures tested (Figure 17b). However, after 9 months at both 4 and 25ºC the content of polyphenols decreased, concomitant with an increase in the content of condensed tannins (Figure 17c), which might suggest polymerization of phenolic compounds during long-term storage.

Regarding the antioxidant capacity of RGPE, based on results obtained with the FRAP assay, there were no significant differences between basal, 3, 6 and 9 months data analysed by the paired test (Figure 17e). However, when assessed using ORAC and ABTS techniques the total antioxidant capacity decreased, particularly after 9 months, in keeping with the decrease of the phenolic content (Figure 17d and 17f). In agreement with the latter results, other authors have described a gradual reduction in the total polyphenol content and antioxidant capacity (evaluated using FRAP and DPPH free-radical scavenging assays) in orange juices stored at 18, 28 and 38°C for 2, 4 and 6 months (Klimczak et al., 2007). In grape supplements stored at 45°C and 75% humidity for 60 days, a progressive decrease in ORAC antioxidant capacity, as far as 43% compared to the initial value, has also been described (Monagas et al., 2005).

As for the effect of storage temperature (4ºC *versus* room temperature), overall there was no effect either on total polyphenol or condensed tannin levels or the antioxidant capacity of RGPE, except for ABTS results which were higher at 25ºC. Regarding ORAC values, antioxidant capacity at 4ºC was higher during the first half of the experiment but decreased over longer storage ($p = 0.012$).

Effect of storage temperature and time on microbiological load

Up to 9 months, the total count of bacteria and enterobacteria were under 10 (colony-forming units) cfu/g, indicating that no microbial growth in the samples during storage took place. Accordingly, *Salmonella* was not detected during 9 month storage. Mold and yeasts were under 100 cfu/g and *E.coli* was less than 10 cfu/g, which were within the accepted values in the samples. All these results indicate that the samples were microbiologically safe and free of pathogenic

bacteria along the experiment and did not undergo any changes during the storage time studied (Table 11).

Table 11. Microbiological analysis of the red grape pomace according to storage time and temperature.

	0	3 months		6 months		9 months	
(cfu/g)		4°C	25°C	4°C	25°C	4°C	25°C
Total aerobic	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$
Enterobacteriaceae	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$
E.coli β-glucuronidase	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$	$< 10^1$
Mould and yeasts	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
Salmonella (presence)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

Effects of in vitro digestion on the phenolic compounds and antioxidant capacity

In this work, we investigated the effects of an *in vitro* gastric and pancreatic digestion on the phenolic compounds and antioxidant capacity in RGP, as well as the changes in the contents of its phenolic components along the digestion process. Polyphenol content and antioxidant capacity of the samples before and after different steps of digestion are shown in Figure 18 and 19, respectively. After *in vitro* gastric digestion, no significant changes were observed in the phenolic content determined by the Folin-Ciocalteu assay, keeping the initial amount (4.80 g/100 g DM) determined in RGP (Figure 18). Likewise, the samples incubated at pH 2 without the enzyme showed similar results (GD-CT). These results were in line with those obtained after evaluating antioxidant activity by FRAP, ABTS and ORAC (Figure 19). No differences were observed after pepsin digestion compared with the initial conditions, although values in the control samples without pepsin tended to be lower. This is in agreement with findings by other authors on the effect of gastric digestion on the total phenolic content and antioxidant activity. No differences in the total phenolic content of pomegranate fresh juice were evident when compared before and after pepsin digestion (292 mg of GAE/L; Pérez-Vicente et al., 2002). Likewise,

Pereira-Caro et al. (2012b) reported that gastric digestion did not modify the antioxidant activity of the phenolic compounds (hydroxytyrosol and hydroxytyrosyl acetate) of virgin olive oil.

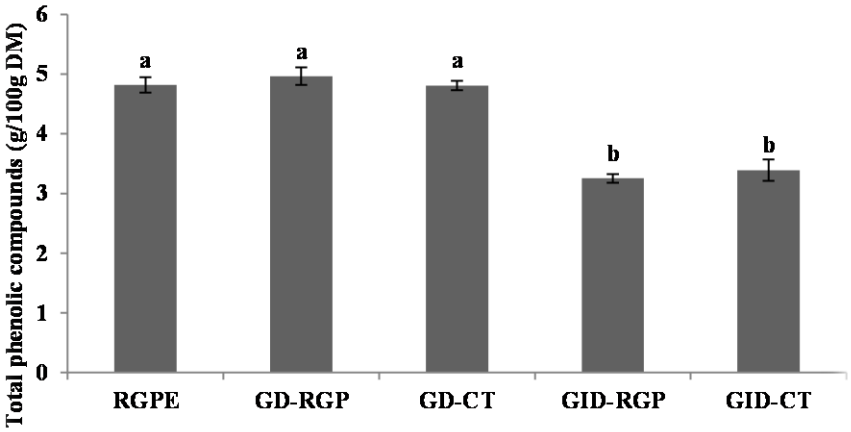


Figure 18. Total polyphenol content of the studied red grape pomace and effect of *in vitro* digestion. DM, Dry matter; GD-RGP, gastric digested red grape pomace; GD-CT, gastric digestion control (with no added pepsin); GID-RGP, gastrointestinal digested red grape pomace; GID-CT, gastrointestinal digestion control (with no added pancreatin and bile salts); RGPE, red grape phenolic extract. Results are means ± SD (n= 4–8). Different letters indicate statistically significant differences ($p < 0.05$).

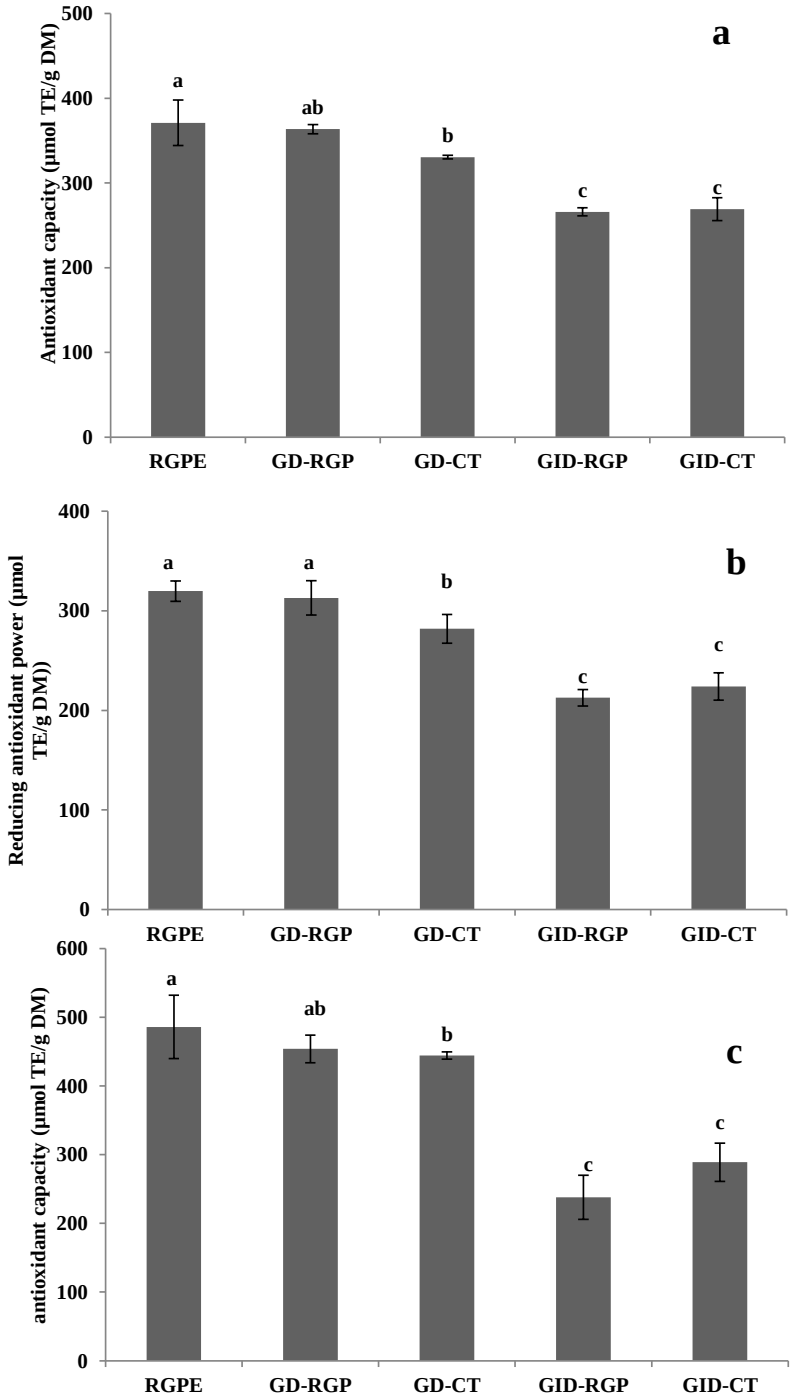


Figure 19. Antioxidant capacity of the red grape phenolic extract and in the digested pomace. (a) ABTS (b) FRAP (c) ORAC. DM, Dry matter; GD-CT, gastric digestion control (with no added pepsin); GD-RGP, gastric digested red grape pomace; GID-CT, gastrointestinal digestion control (with no added pancreatin and bile salts); GID-RGP, gastrointestinal digested red grape pomace; RGPE, red grape phenolic extract; TE, Trolox equivalents. Results are means ± SD (n= 4–8). Different letters indicate statistically significant differences ($p < 0.05$).

Conversely, after the pancreatic stage a significant decrease was observed in the total polyphenol content (3.11 g/100 g DM) and, accordingly, in the antioxidant activity (Figures 18 and 19). The antioxidant activity measured with ABTS was 265.9 µmol TE/g DM compared to 371.3 µmol TE/g DM before digestion procedure. The antioxidant activity determined with FRAP and ORAC assays also showed a similar trend with respect to that measured with the ABTS assay during gastric-intestinal digestion. Again, this is in line with previously reported data, since pancreatic digestion also reduced hydroxytyrosol and hydroxytyrosyl acetate phenolic compounds and antioxidant activity (Pereira-Caro et al., 2012b). Accordingly, green and black tea showed a major reduction in polyphenol concentration (28 and 36% loss in green and black tea, respectively) and in antioxidant activity measured by the FRAP assay (27 and 29% loss in green and black tea, respectively) at the slightly alkaline pH during intestinal digestion (Record and Lane 2001). These results show a clear correlation between changes in polyphenol concentration and antioxidant activity during digestion. Since results were similar in the control without digestive enzymes and bile salts (GID-CT) (Figures 18 and 19) and in the control with pancreatin and no bile salts (data not shown), it can be inferred that the enzymes and bile salts did not influence the reduction of antioxidant capacity and phenolic content, indicating the modifications observed were mostly due to the slightly alkaline pH during pancreatic digestion.

The content of different phenolic acids, flavonols and anthocyanins after *in vitro* digestion is shown in Table 9. According to the HPLC-DAD results, gastric digestion had no significant effect on most of the major phenolic compounds in RGP, and only slight reductions in the content of some phenolic acids were seen, although the total phenolic acid content was not statistically different from the sample prior to pepsin digestion. Flavonoids were slightly more susceptible to the gastric digestion, since most flavonols and all anthocyanidins but peonidin-3-*O*-glucoside showed losses during pepsin treatment, especially relevant in the case of anthocyanins. However, since phenolic acids are the major components of the RGP, overall the gastric digestion did not affect the total phenolic content (Table 9).

In contrast, phenolic compounds were significantly altered during the pancreatic digestion. A decrease in phenolic acids, flavonols and anthocyanins were observed during the pancreatic digestion (9, 34 and 100%, respectively),

although the higher abundance of phenolic acids than anthocyanins and flavonols compensated their lost, which accounted for 14% all together. Losses of the main phenolic acids varied from just 4% of gallic acid to up to 21% of vanillic acid. Regarding flavonols, quercetin and its main derivatives, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide diminished 46, 25, and 28% respectively, after gastrointestinal digestion, although myricetin was the most labile flavanol, with a loss of 67% after the *in vitro* intestinal digestion. On average, up to 33% of the total flavonols in RGP were lost after gastrointestinal digestion (Table 9). These results are in agreement with previously published data, since quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide, as the most abundant flavonols in *V. vinifera* leaves, decreased 38% and 40%, respectively (Sangiovanni et al., 2015). Similarly, flavonols in chokeberry juice were also partially degraded under *in vitro* intestinal conditions, with losses of individual compounds ranging between 15% and 30% (Bermudez-Soto et al., 2007). Regarding anthocyanins, the concentration of different anthocyanins dramatically decreased after gastrointestinal digestion, with final concentrations below the limit of detection. Anthocyanins are not stable and are largely affected by the alkaline conditions during pancreatic digestion. Other authors also reported a high decrease of anthocyanins after *in vitro* digestion (Pérez-Vicente et al., 2002; McDougall et al., 2005). The anthocyanins concentration of *Vitis vinifera* leaves dramatically decreased, with particularly high reductions for delphinidin-3-*O*-glucoside and malvidin-3-*O*-glucoside (around 70% reduction) after gastrointestinal digestion (Sangiovanni et al., 2015). Our results, in accordance with previously published data, show that dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine.

Overall, most of the dietary polyphenols examined are stable under gastric conditions and are lost during incubation with pancreatic-bile salts at neutral or slightly alkaline pH. The fact that the absence of enzymes and bile salts in the controls did not influence the modification of the antioxidant capacity and total polyphenol content during the *in vitro* digestion suggests that the physiological pH environment present in the small intestine reduced polyphenols' digestive stability. These dietary polyphenols can be transformed into other unknown and/or undetected structural forms, with different chemical properties and consequently, different bioaccessibility, bioavailability and biological activity in the small intestine (Bermudez-Soto et al., 2007).

Conclusions

RGP presented high antioxidant activity and thus potential as a functional ingredient. Storage of the product at either 25 or 4ºC up to 6 months produced minimal degradation of phenolic compounds and loss of antioxidant activity. However, after 9 months these parameters decreased concomitant with an increased content in condensed tannins. This could be related with the changes in colour observed, particularly at 4ºC. From a microbiological point of view, RGP may be stored safely at the temperatures studied for as long as 9 months. Polyphenols contained in RGP were highly stable under conditions mimicking those in the stomach and were more sensitive to the mild alkaline conditions in the small intestine, their content in RGP being slightly reduced. According to *in vitro* digestion and storage results, the red grape pomace presents high bioactive potential.

Study 2

A phenolic extract from grape by-products and its main hydroxybenzoic acids protect Caco-2 cells against pro-oxidant induced toxicity.

Summary

Grape/wine industry produces large amounts of by-products, however knowledge on their health-promoting qualities is limited. This study investigated the effects of a grape phenolic extract (GPE) and its phenolic compounds, gallic acid (GA) and syringic acid (SA) on human intestinal Caco-2 cells, directly or after cytotoxicity induced by *tert*-butylhydroperoxide (*t*-BOOH). Direct treatment with 0.1-10 μg/mL of GPE, or 0.1-10 μM of GA and SA produced no major cytotoxic effect, neither changes in antioxidant defenses (glutathione content, glutathione peroxidase and reductase activities) nor protein damage (carbonyl groups). However, 10 μg/mL GPE, 1 and 10 μM GA and 10 μM SA decreased reactive oxygen species (ROS) production.

Pre-treatment with GPE, SA and GA at the same concentrations for 20 h showed that 10 μg/mL GPE and 10 μM GA or SA significantly counteracted ROS increase induced by *t*-BOOH. 10 μg/mL GPE and 1-10 μM of GA or 10 μM of SA significantly reduced prooxidant-induced cytotoxicity. 1-10 μg/mL of GPE, 1-10 μM of GA and 10 μM SA significantly recovered both depleted glutathione and enhanced glutathione reductase and peroxidase activities, and reduced protein oxidative damage. Therefore, treatment with realistic concentrations of GPE and its main hydroxybenzoic acids protected Caco-2 cells against induced oxidative stress.

Keywords: antioxidant, Caco-2 cells, gallic acid, grape by-product, oxidative stress, syringic acid.

Introduction

Grape (*Vitis vinifera* L.), wine and winery by-products have great economic value. Italy, France, Spain, and the United States are among the most important producers in the world. Approximately 80% of the grapes are used in winemaking and ~20% of the weight of processed grapes remains as by-products annually generating about 10 million tons of waste from wineries (Kammerer et al., 2004). Use of by-products from wine industry is becoming a hot topic; they are employed in juices, jam, yogurts, jelly and other foods as colorants and a source of dietary fiber. In the last years, grape by-products have also been commercialised as nutraceuticals or dietary supplements (Georgiev et al., 2014). The composition of these products includes phenolic compounds such as phenolic acids, flavonoids, procyanidins and stilbenes (Yu and Ahmedna, 2013). Numerous studies have demonstrated that foods rich in phenolic compounds exert beneficial health effects as it has been reviewed (Bravo, 1998; Tomás-Barberán and Andrés-Lacueva, 2012), being the antioxidant activity one of their most important biological properties. The reduction of oxidative stress by grape by-products has been reported in animals (Choi et al., 2012). Ten day pretreatment of rats with a grape seed proanthocyanidin extract decreased DNA oxidative damage in isolated colonocytes (Giovanelli et al., 2000). Due to the "French paradox" the majority of the studies have been focused on flavonoids, being hydroxybenzoic acids (also a major component of grape wine by-products) much less studied or known.

Intestinal epithelium is exposed to toxicity induced by luminal oxidants from ingested foods which can cause oxidative damage to macromolecules and tissues, and as a consequence bowel discomfort and ultimately disease. This fact supports the interest of studying oxidative stress-induced toxicity and the effect of dietary antioxidants in the intestinal epithelium. Human Caco-2 cell line, derived from human colon cancer cells, is widely used for biochemical and nutritional studies as a cell culture model of human colonocytes since they retain their morphology and most of their function in culture (Rodríguez-Ramiro et al., 2011). Under certain culture conditions, Caco-2 cells can form spontaneously polarized microvillus, closely connected with differentiation characteristics mimicking intestinal epithelium (Artursson and Karlsson, 1991; Yamashita et al., 2000). For this reason, Caco-2 cells have been used to study the intestinal absorption of food components such as peptides (Amigo-Benavent et al., 2014) and phenolic

compounds (Konishi and Kobayashi, 2004; Mateos et al., 2011), among others. This cell model has also been previously employed in our group to test the protective effect of cocoa flavanols against the damage of food toxic acrylamide (Rodriguez-Ramiro et al., 2011) and the modulation of oxidative status by dietary flavanols (Ramos et al., 2011). Bearing this in mind, the aim of this work was to investigate the protective effect of a grape phenolic extract (GPE) derived from wine by-products and its main hydroxybenzoic compounds against toxicity induced by prooxidant *tert*-butylhydroperoxide (*t*-BOOH) in Caco-2 cells.

Results

Characterisation and quantification of phenolic compounds in GPE

Figure 20 shows HPLC profiles of GPE at 280 (A), 360 (B) and 520 (C) nm. The peaks were identified by HPLC-ESI-QToF analysis and comparison of retention time and UV spectrum with commercial standards of phenolic compounds. Up to 28 different phenolic compounds were detected in GPE. Table 12 shows the list of compounds along with their retention time, UV characteristics of the chromatographic peaks, molecular ion, fragment ions and molecular formula (M.F.), in addition to the quantitative analysis. According to the chromatographic analysis, the total phenolic content in GPE was 2211.6 μg/g dry matter, mostly phenolic acids (1877.1 μg/g) with lower content of flavonols (305.5 μg/g) and anthocyanins (29.0 μg/g). The major phenolic acids were gallic acid (GA) and ellagic acid. Among flavonols, quercetin was the most abundant. Due to the difficult solubilisation of ellagic acid, GA was chosen to carry out the cell culture experiments. Syringic acid (SA; 4-hydroxy-3,5-dimethoxybenzoic acid) was also selected on the basis of its abundance and to evaluate the biological effect that its double methyl substituents presents, in comparison with GA (3,4,5-trihydroxybenzoic acid). Although the quercetin content was similar to that of SA, it was not selected as this compound has been extensively studied in our group and has shown to strongly protect HepG2 cells against oxidation induced by *tert*-butylhydroperoxide (Alía et al., 2005; Alía et al., 2006, among others).

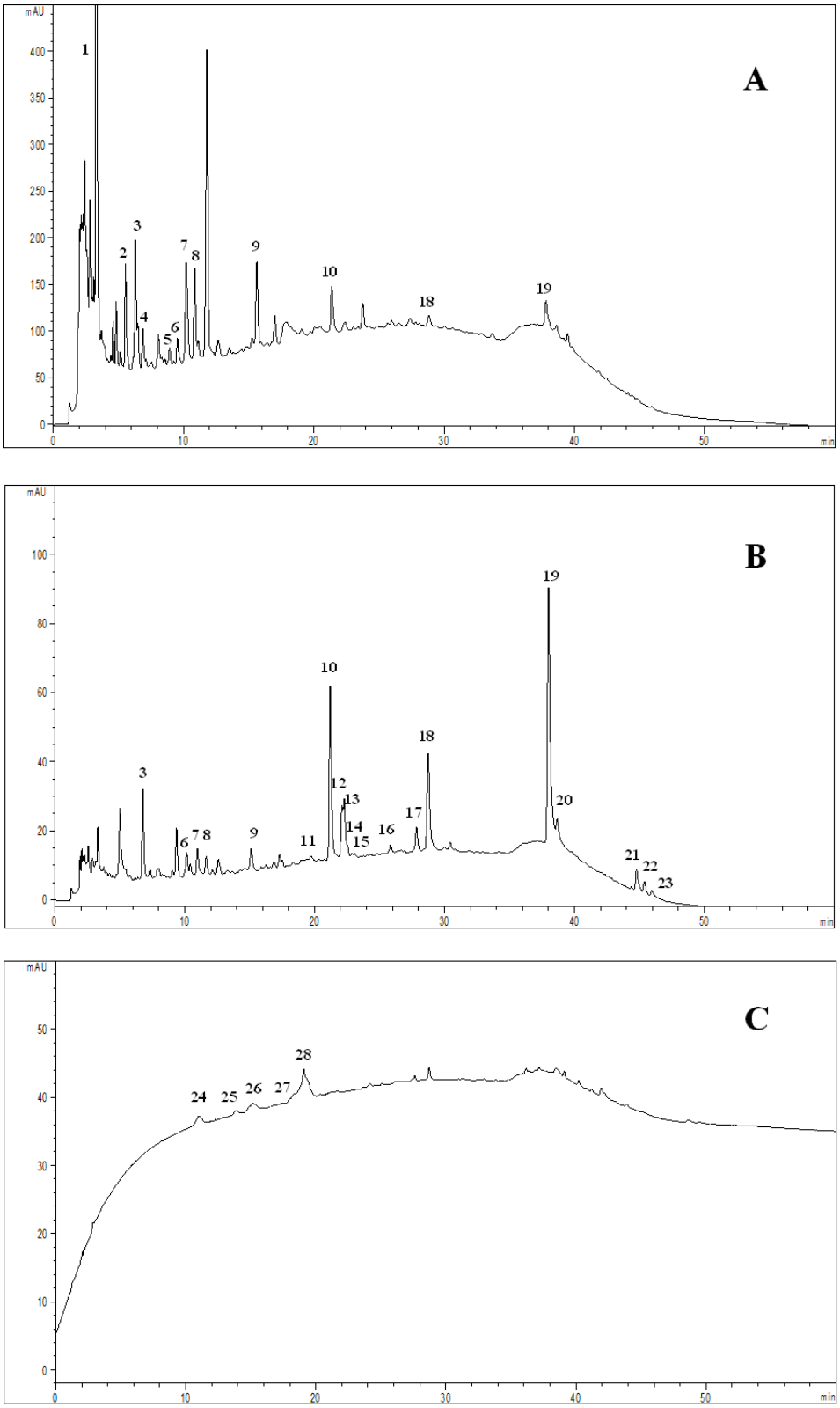


Figure 20. HPLC chromatogram profiles recorded at 280 (A), 360 (B) and 520 (C) nm of the grape phenolic extract. Peaks were identified by HPLC–ESI-QToF analysis and quantified by HPLC-DAD.

Table 12. Content of phenolic compounds identified in GPE by HPLC-DAD in combination with LC-QTOF analysis.

Chr. Peak	Compound	RT (min)	λmax	Molecular Ion	Fragment ions	M.F.	µg/g d.m.
Phenolic acids (280 nm)							
1	Gallic acid	3.3	272	169.0142	125	$C_7H_6O_5$	627.3 ± 63.1
2	Protocatechuic acid	5.5	260, 292	153.0193	109	$C_7H_6O_4$	231.0 ± 19.7
3	Caftaric acid	6.3	296, 324	311.0409	179	$C_{13}H_{12}O_9$	109.9 ± 7.7
4	*p*-hydroxybenzoic acid	6.5	285	137.0244	93	$C_7H_6O_3$	60.6 ± 2.3
5	*Cis*-coutaric acid	9.5	294, 309	295.0459	163	$C_{13}H_{12}O_8$	11.7 ± 1.2
6	*Trans*-coutaric acid	9.8	295, 314	295.0459	163	$C_{13}H_{12}O_8$	20.4 ± 2.3
7	Fertaric acid	10.2	297, 331	325.0565	193	$C_{14}H_{14}O_9$	162.3 ± 10.7
8	Vanillic acid	10.8	260	167.0350	123	$C_8H_8O_4$	126.7 ± 20.2
9	Syringic acid	15.6	279	197.0455	153, 182	$C_9H_{10}O_5$	102.5 ± 4.1
10	Ellagic acid	21.3	254, 368	300.9990		$C_{14}H_6O_8$	424.6 ± 35.6
Total phenolic acids							**1877.1 ± 166.9**
Flavonols (360 nm)							
11	Rutin	19.8	256, 354	609.1461	301	$C_{27}H_{30}O_{16}$	9.0 ± 0.8
12	Quercetin-3-*O*-glucoside	22.1	256, 354	463.0882	301	$C_{21}H_{20}O_{12}$	21.2 ± 2.1
13	Quercetin-3-*O*-glucuronide	22.2	254, 353	477.0675	301	$C_{21}H_{18}O_{13}$	25.1 ± 0.8
14	Miricetin-3-*O*-glucoside	22.9	258, 358	479.0831	317	$C_{21}H_{20}O_{13}$	1.7 ± 0.1
15	Isoramnetin-3-*O*-glucoside	23.1	254, 354	477.1038	315	$C_{22}H_{22}O_{12}$	8.4 ± 1.1
16	Kampherol-3-*O*-glucoside	25.8	264, 348	447.0933	285	$C_{21}H_{20}O_{11}$	21.8 ± 2.3
17	Siringetin-3-*O*-glucoside	27.8	252, 360	507.1144	345	$C_{23}H_{24}O_{13}$	17.1 ± 2.0
18	Miricetin	28.7	254, 368	317.0303		$C_{15}H_{10}O_8$	34.8 ± 3.3
19	Quercetin	38.1	255, 370	301.0354		$C_{15}H_{10}O_7$	107.8 ± 6.2
20	Laricetrin	38.7	250, 372	331.0459		$C_{16}H_{12}O_8$	16.5 ± 1.4
21	Kaempherol	44.8	264, 363	285.0405		$C_{15}H_{10}O_6$	19.3 ± 6.4
22	Isoramnetin	45.4	256, 368	315.0510		$C_{16}H_{12}O_7$	11.5 ± 1.0
23	Siringetin	46.0	254, 374	345.0616		$C_{17}H_{14}O_8$	11.0 ± 0.5
Total flavonols							**305.5 ± 27.9**
Anthocyanins (520 nm)							

24	Delphinidin-3-*O*-glucoside	10.7	520	465.1028	303	$C_{21}H_{20}O_{12}$	4.1 ± 0.2
25	Cyanidin-3-*O*-glucoside	14.2	510	449.1078	287	$C_{21}H_{20}O_{11}$	2.0 ± 0.2
26	Petunidin-3-*O*-glucoside	14.9	528	479.1184	317	$C_{22}H_{23}O_{12}$	4.0 ± 0.2
27	Peonidin-3-*O*-glucoside	18.1	520	463.1235	301	$C_{22}H_{23}O_{11}$	2.3 ± 0.2
28	Malvidin-3-*O*-glucoside	19.0	522	493.1341	331	$C_{23}H_{25}O_{12}$	16.6 ± 1.1
Total Anthocyanins							**29.0 ± 1.9**
Total Phenols							**2211.6 ± 196.7**

Results expressed as mean ± standard deviation (n=3).

Chr. Peak: chromatographic peak; d.m.: dry matter; M.F.: molecular formula; RT: retention time.

Viability, cytotoxicity and antioxidant effects of GPE, GA and SA on Caco-2 cells in basal conditions

Table 13 shows the results obtained in basal conditions after direct treatment of Caco-2 cells with the GPE and the two specified compounds. No statistically significant effects were found in cell viability determined by WST-1 reagent, except for 10 µM of GA which showed a percentage of viability of 89%. The percentage of LDH when cells were incubated with 0.1-10 µg/mL GPE and 0.1-10 µM GA or SA was similar to control cells, except for 10 µM of GA which was slightly decreased. High LDH values indicate a cytotoxic effect; therefore, it can be assumed that the range of concentrations finally selected (0.1-10 µM) does not induce cytotoxic effects in Caco-2 cells. This was an essential point to test before looking into the potential nutraceutical and/or therapeutical properties of these compounds.

Table 13. Direct effect of GPE, GA and SA on Caco-2 cell viability, antioxidant defenses (GSH, GPx and GR activities) and protein damage (carbonyls).

	Cell viability %	**LDH** %	**GSH** ng	**GPx** mU/mg protein	**GR** mU/mg protein	**Carbonyl** nmol /mg protein
Control	100.0±3.9^{a}	17.9±1.5^{a}	244.3±14.5^{a}	104.1±12.5^{a}	2.8±0.2^{a}	2.1±0.4^{a}
GPE						
0.1µg/mL	93.4±6.1^{ab}	18.5±1.0^{a}	240.8±10.6^{a}	100.2±9.4^{a}	2.8±0.2^{a}	1.9±0.3^{a}
1 µg/mL	91.6±4.3^{ab}	17.9±0.8^{a}	233.8±6.4^{a}	104.6±5.4^{a}	2.7±0.1^{a}	2.3±0.3^{a}
10 µg/mL	91.8±4.3^{ab}	16.9±1.7^{a}	248.6±14.2^{a}	89.1±4.2^{a}	2.6±0.3^{a}	2.3±0.2^{a}
GA						
0.1µM	100.2±3.5^{a}	16.2±1.2^{a}	241.0±16.4^{a}	101.8±5.5^{a}	2.9±0.2^{a}	1.8±0.2^{a}
1 µM	96.6±5.7^{ab}	17.1±0.6^{a}	248.7±20.3^{a}	108.6±2.5^{a}	2.6±0.3^{a}	2.0±0.2^{a}
10 µM	88.9±2.6^{b}	16.0±0.6^{a}	236.9±2.5^{a}	102.6±2.6^{a}	2.5±0.3^{a}	2.0±0.1^{a}
SA						
0.1 µM	93.6±4.8^{ab}	16.3±1.2^{a}	228.7±17.4^{a}	95.0±8.1^{a}	2.6±0.1^{a}	2.0±0.2^{a}
1 µM	94.1±7.1^{ab}	16.2±2.0^{a}	221.1±17.9^{a}	98±1.1^{a}	2.8±0.1^{a}	2.0±0.1^{a}
10 µM	92.0±4.3^{ab}	16.4±1.7^{a}	229.4±13.4^{a}	90.0±8.5^{a}	2.7±0.1^{a}	2.2±0.1^{a}

Results expressed as mean ± standard deviation (n = 4-8). Different letters within a column denote statistically significant differences ($p<0.05$).

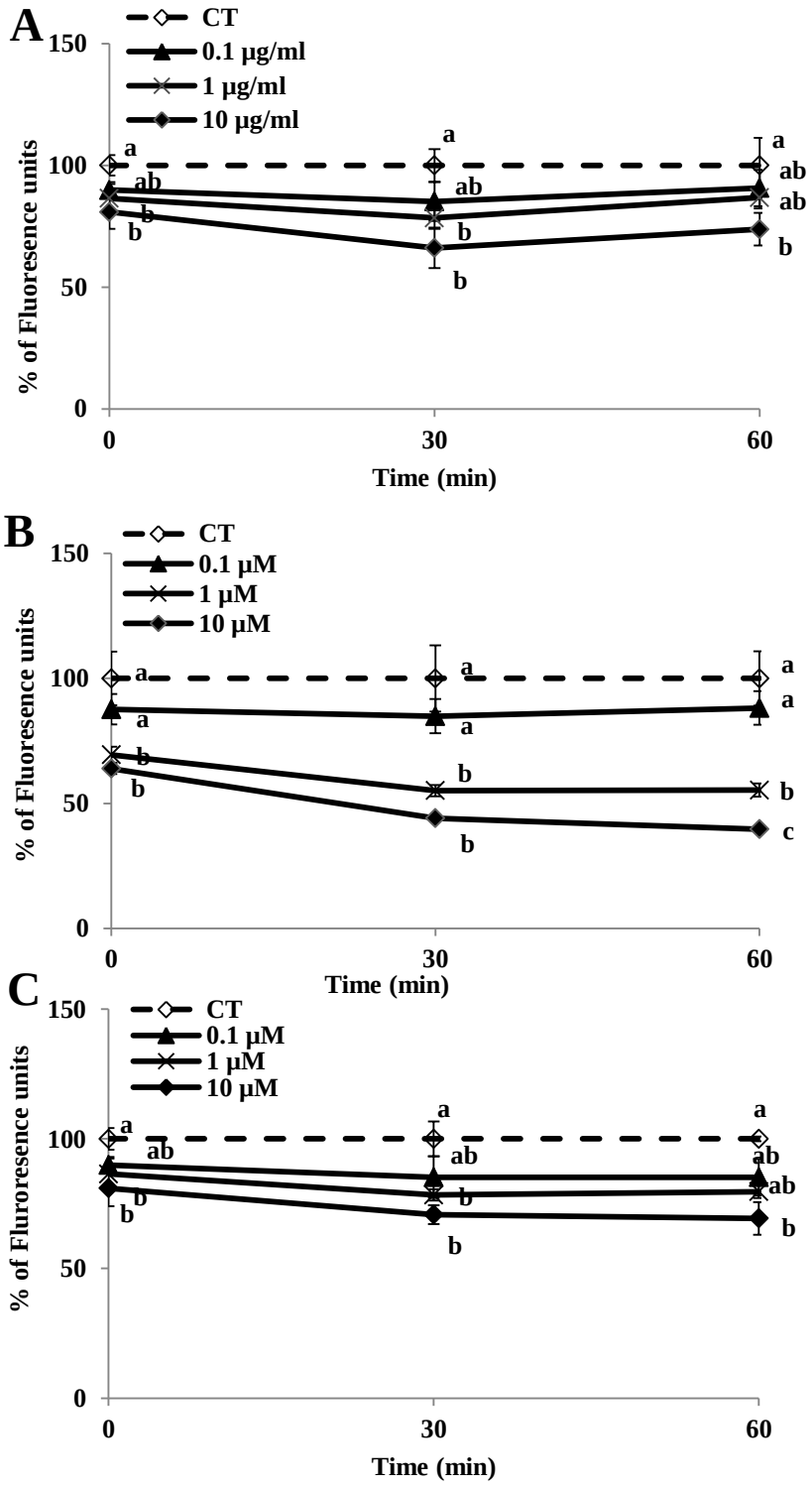


Figure 21. Direct effect on Caco-2 cell intracellular ROS generation after treatment with GPE (A), SA (B) and GA (C) over 60 min. Results are expressed as % of fluorescence arbitrary units against time (n=4–6). Different letters denote statistically significant differences within the same time period ($p<0.05$).

Likewise, there were no statistically significant changes in cell antioxidant defenses (GSH content, GPx and GR activities) and protein oxidation after 20 h of treatment with these samples. Thus, it can be assumed that this range of concentration can be safely used to study the protective effect of GPE, GA and SA against a condition of oxidative stress on cells. Moreover, incubation of Caco-2 cells with 1-10 µM SA and 10 µg/mL GPE or 10 µM of GA for 1 h statistically decreased ROS production (Figure 21).

Protective effect of GPE, GA and SA on Caco-2 cells in a condition of oxidative stress

The protective effect of pre-treatment with 0.1, 1 and 10 µg/mL GPE, or 0.1, 1 and 10 µM GA and SA for 20 h in Caco-2 cells against oxidative stress induced by *t*-BOOH treatment was studied. In order to generate a condition of cellular oxidative stress, cells were treated with 400 µM *t*-BOOH, a strong pro-oxidant, and ROS were evaluated for 1 h of *t*-BOOH exposure, and cytotoxicity and antioxidant defences after 3 h of oxidative insult. In these experiments, a negative control with *t*-BOOH-untreated cells and a positive control of oxidative stress in cells with the same amount of *t*-BOOH and for the same incubation time were included.

Cell toxicity

Treatment with 400 µM *t*-BOOH significantly enhanced (3.5 times compared with negative control) leakage of LDH, indicating prominent cell damage in Caco-2 cells at 3 h (Figure 22). However, when cells were pre-treated with 10 µg/mL GPE, 1-10 µM GA and 10 µM SA a significant decrease in cytotoxicity induced by *t*-BOOH was observed compared with the stressed control.

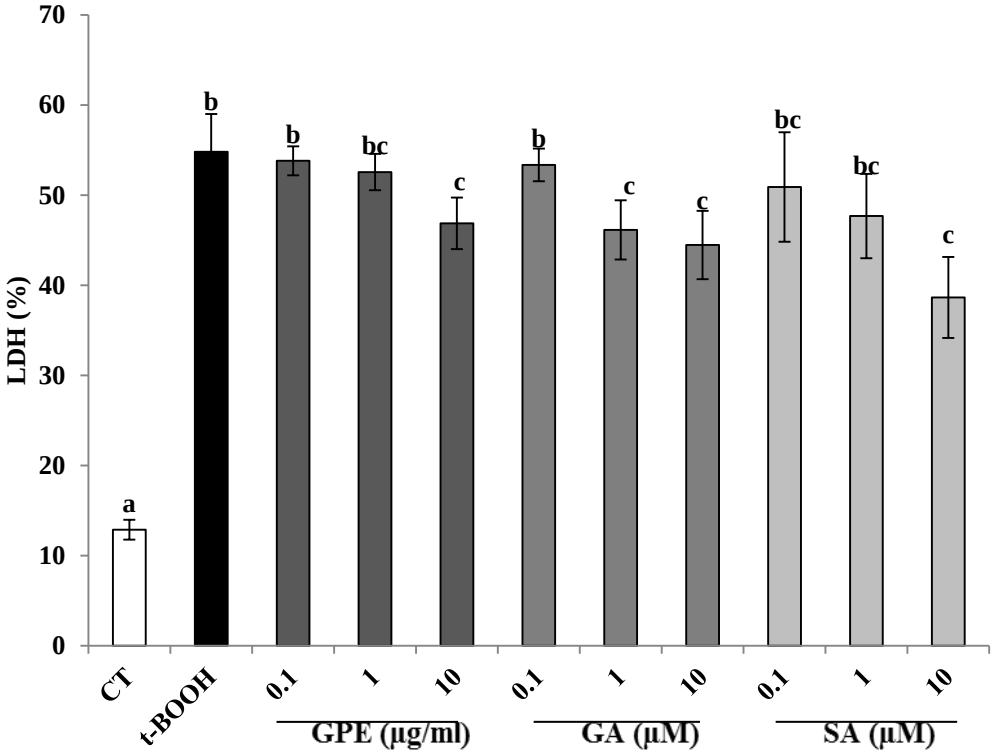


Figure 22. Protective effect of pre-treatment with GPE, GA and SA for 20 h on cell toxicity (LDH %) against oxidative stress induced by *t*-BOOH for 3 h. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences ($p<0.05$).

ROS generation

Caco-2 cells treated with *t*-BOOH for 1 h showed a two-fold increase in intracellular ROS generation as compared to non-stressed controls. Treatment of cells with 10 µg/mL GPE and 1-10 µM GA or SA significantly decreased ROS production compared to that of stressed cells (Figure 23). Lower concentrations of the tested compounds did not show statistical differences compared with *t*-BOOH control cells.

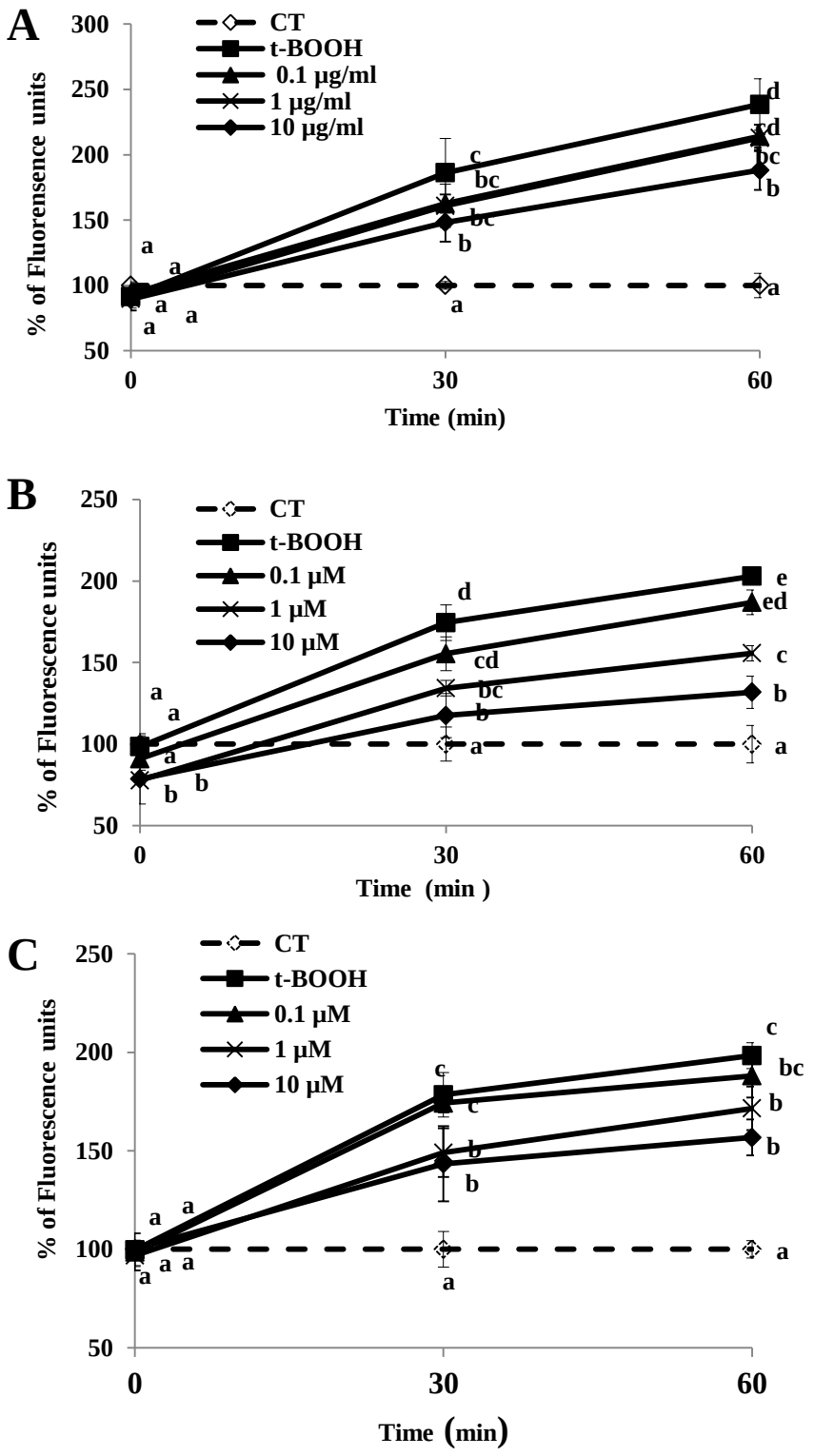


Figure 23. Protective effect on Caco-2 cell intracellular ROS production pre-treated with the noted concentrations of GPE (A), SA (B) and GA (C), and after *t*-BOOH exposure for 60 min. Results are expressed as % of fluorescence arbitrary units against time (n = 4–6). Different letters within the same time denote statistically significant differences ($p<0.05$).

Antioxidant defences

Non-enzymatic defences: Addition of 400 µM *t*-BOOH to Caco-2 cells for 3 h evoked a dramatic decrease in the cytoplasmic glutathione (GSH; ng/mg protein) to 50% of non-stressed cells (Figure 24). Pre-treatment with 1-10 µg/mL GPE, 1-10 µM GA and 10 µM SA partly recovered GSH levels.

Enzymatic defences: Oxidative insult with 400 µM *t*-BOOH in Caco-2 cell culture induced a ~2-fold increase in the enzyme activities of glutathione peroxidase (GPx) and glutathione reductase (GR) (Figure 25). When cells were pre-treated with 1-10 µg/mL GPE and 1-10 µM GA or SA for 20 h, the chemically-induced rise in GPx activity was prevented or reduced compared with stressed cells. GR over-activation was also restricted compared with *t*-BOOH treated cells after pre-incubation with 1-10 µg/mL GPE and 1-10 µM of either phenolic compound. Lower concentrations of GPE, GA and SA did not significantly decrease GPx and GR activities.

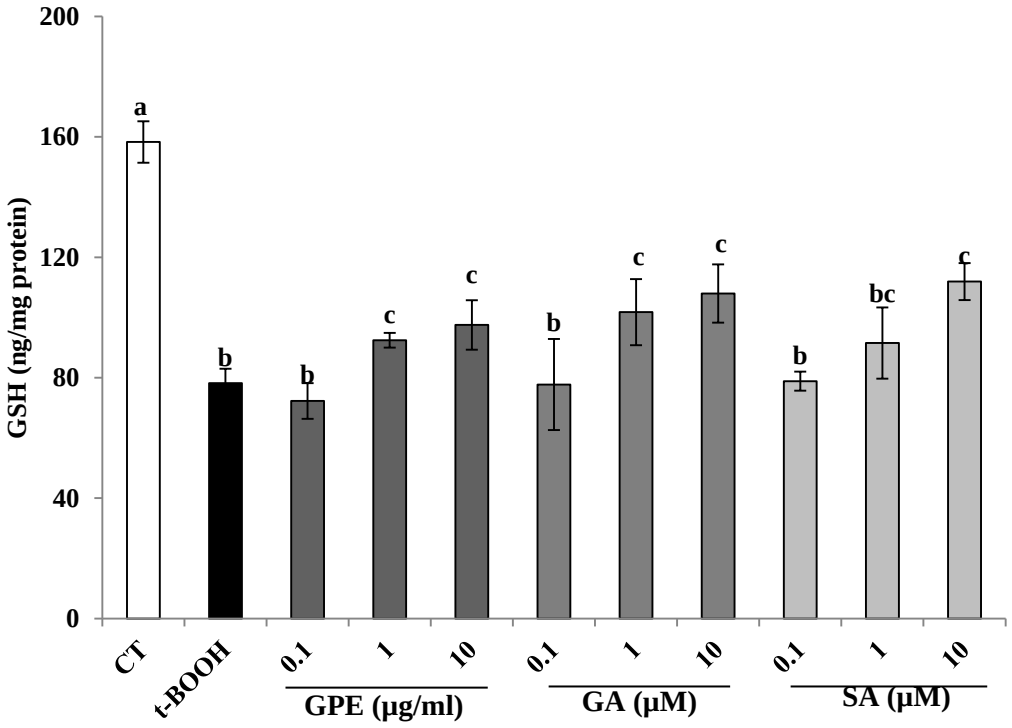


Figure 24. Protective effect of pre-treatment with GPE, GA, SA for 20 h, and after *t*-BOOH exposure for 3 h on GSH content. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences (p<0.05).

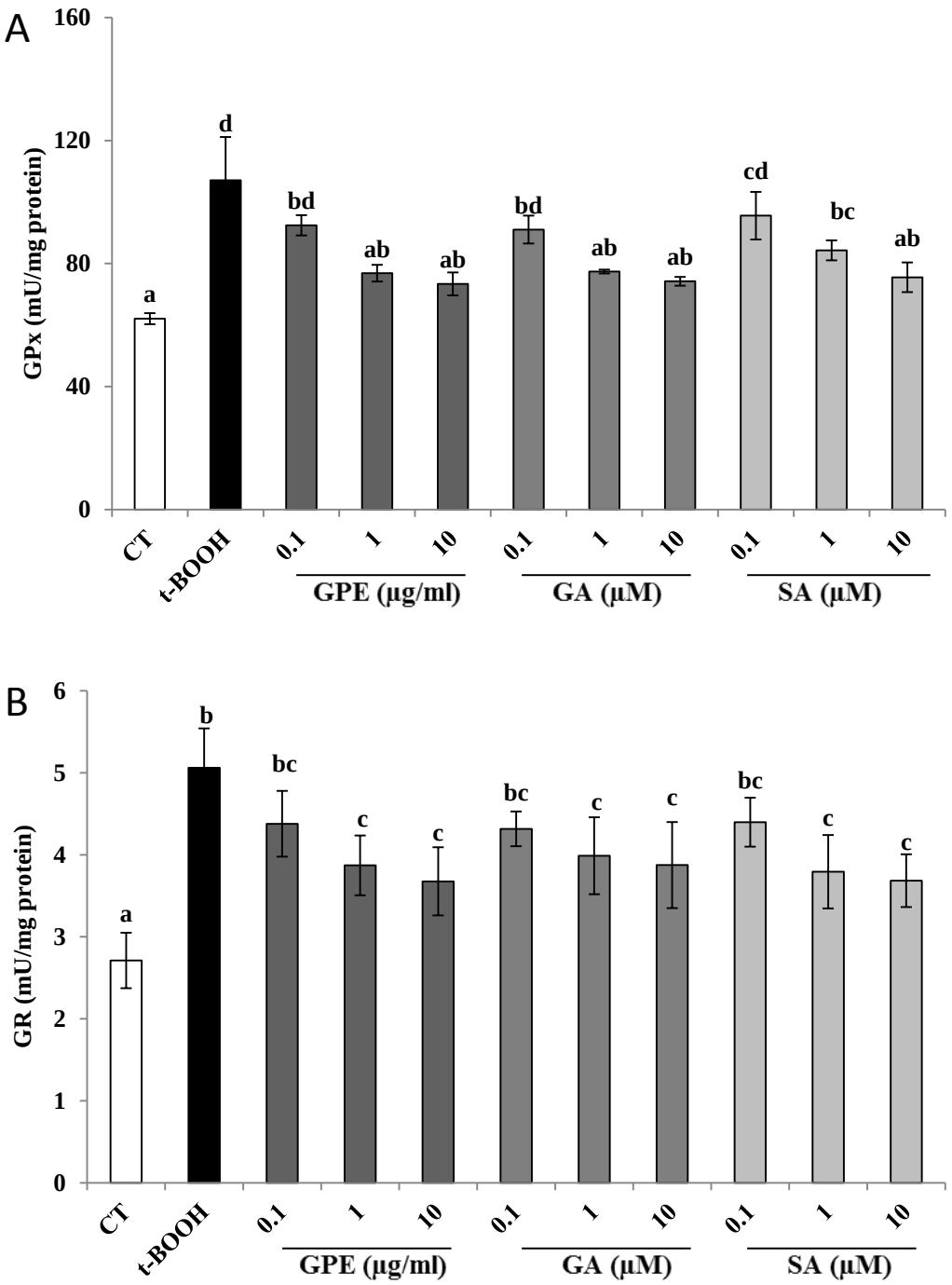


Figure 25. Protective effect of pre-treatment with GPE, GA and SA for 20 h, and after *t*-BOOH oxidative insult for 3 h on Caco-2 cell antioxidant enzymes GPx (A) and GR (B). Results are means ± SD (n= 4–8). Different letters denote statistically significant differences ($p<0.05$).

Biomarker of protein oxidative damage

Caco-2 cells treated with 400 µM *t*-BOOH for 3 h showed a significant increase in the cellular concentration of carbonyl groups, indicating extensive oxidative damage to cell proteins (Figure 26). Pre-treatment of Caco-2 cells with 1-10 µg/mL GPE, 0.1-10 µM GA and SA for 20 h significantly prevented the increase of carbonyl groups induced by *t*-BOOH, indicating a reduced level of protein oxidation in response to the stressor.

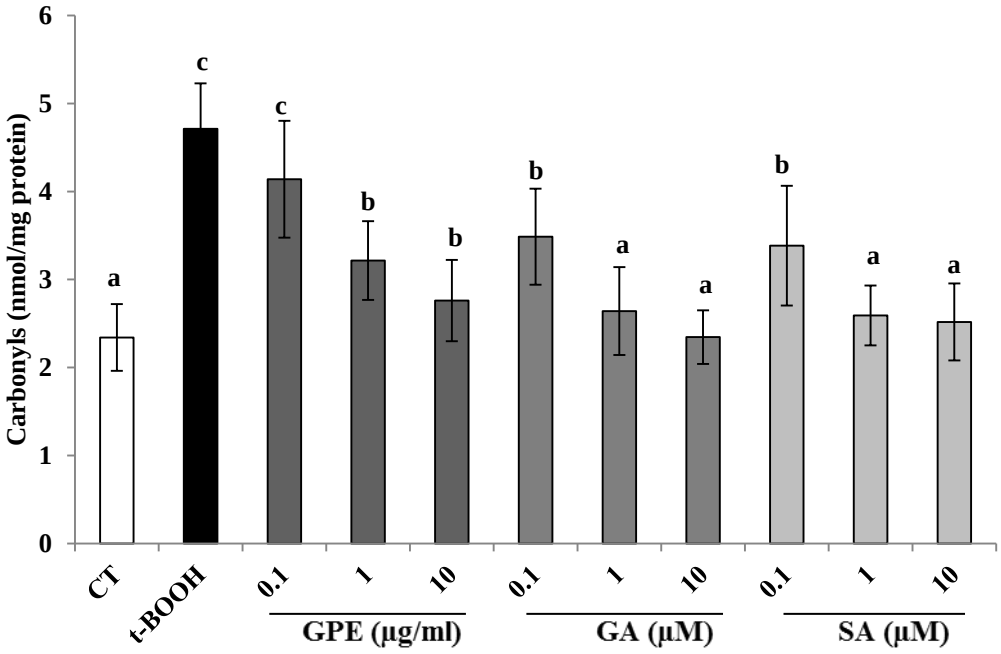


Figure 26. Protective effect of pre-treatment with GPE, GA and SA against oxidative stress induced by *t*-BOOH for 3 h on the Caco-2 cell protein-oxidation (carbonyls groups). Results are means ± SD (n=4–8). Different letters denote statistically significant differences (p<0.05).

Discussion

In this work, the phenolic composition of a wine by-product has been investigated together with the protective antioxidant effects of its phenolic extract and its major hydroxybenzoic acids, GA and SA, in Caco-2 cells.

The HPLC analysis showed that hydroxybenzoic acids were the most abundant phenolic compounds in GPE followed by flavonols and hydroxycinnamic acids, with lower amounts of anthocyanins. The presence of

vanillic, gallic, and protocatechuic acids agreed with Obreque-Slier et al. (2010) and Rodríguez-Montealegre et al. (2006) for grape pomace. Flavonol content was similar to the concentration described in red grape pomace (Kammerer et al., 2004) and dietetic supplements derived from grape skin and stalks (Monagas et al., 2006).

Previous studies have demonstrated that wine by-products may have beneficial effects on diabetes, cancer and other diseases mediated by oxidative stress (Shrikhande, 2000). Their use as antioxidants against rancidity and bacterial pathogens has also been reported (García-Lomillo et al., 2014). The biological actions of main phenolic compounds in wine by-products such as hydroxybenzoic acids have been ascribed to their antioxidant capacity, i.e. free radical scavenging and chelation of redox active metal ions (Tomás-Barberán and Andrés-Lacueva, 2012). These phenolic compounds may have potent antioxidant effects *in vitro* and *in vivo*, both in cell cultures and in animals (Chen et al., 2007; Tung et al., 2009; Lakshmi et al., 2014; Yonguc et al., 2015). SA has also shown protective effects in mice with CCl_4-induced liver injury (Itoh et al., 2010). However, there is no indication of the possible chemo-protective effect of hydroxybenzoic acids in colon; therefore, we have conducted the present study employing GPE, a wine by-product and its major hydroxybenzoic acids, GA and SA, in cultured colon-derived cells, in an attempt to unravel the intimate cellular mechanisms involved in the chemo-protection, as well as their potential role as regulators of antioxidant defences.

Elevated doses of these dietary compounds can also act as pro-oxidants in cell culture systems and evoke cellular damage (Forester and Waterhouse, 2010; Halliwell, 2014). Previous studies have shown that high doses of GA (>100 µM) in the cell media could convert O_2 to hydrogen peroxide, which increases ROS and induces selective cytotoxicity for cancer cells but presents much lower toxicity for normal cells (Inoue et al., 1995; Isuzugawa et al., 2001; Forester and Waterhouse, 2010). Therefore, it was necessary to ensure that no direct cell damage was caused by concentrations within the physiological range of the tested antioxidant before investigating its protective effect (Sarriá et al., 2012). Doses of 10-40 µM hydroxycinnamic acids and 10-50 µg/mL of green coffee polyphenolic extract have shown protection against oxidative conditions in HepG2 cells (Baeza et al., 2014). The concentration range used for this study is not far from realistic in order to evaluate the effect at the biological level. A

concentration of 4 μM of GA in human plasma has been observed after consumption of 50 mg of pure gallic acid (Manach et al., 2005). Likewise, 239 μM of GA has been reported in rat plasma after the consumption of 100 mg/kg of grape seed polyphenol extract (Ferruzzi et al., 2009). In the present study, only the highest GA concentration evoked a slight decrease in cell viability after 20 h and most interestingly, 10 μg/mL GPE, 10 μM GA and 1-10 μM SA induced a significant reduction in ROS generation. Moreover, antioxidant defences such as GSH, GPx and GR were not altered by any treatment. Therefore, cells treated with GPE, GA or SA seem to be in a favourable condition to face an oxidative challenge.

The pro-oxidant *t*-BOOH has been extensively used to induce cytotoxicity by oxidative stress in cell culture experiments. It can induce epithelial cell damage, mainly by inhibiting cell viability, inducing cell apoptosis, increasing protein peroxidation and inflammatory response, reducing cellular antioxidant capacity, and increasing intracellular ROS levels (Alia et al., 2006; Martín et al., 2010; Baeza et al., 2014). Before starting the study, several concentrations of *t*-BOOH were tested to assure that toxicity by oxidative stress on Caco-2 cells was produced. Results show that concentrations over 400 μM evoke significant cell damage in the medium and over 40% cell death measured by the crystal violet assay (data not shown). Cells treated with *t*-BOOH also showed remarkably high LDH leakage, but those pre-treated with 10 μg/mL GPE, 10 μM SA or 1-10 μM GA exhibited a significant decrease in *t*-BOOH cytotoxicity. This result indicated that the integrity of the challenged cells was remarkably protected against the potent oxidative insult.

ROS production is a good indicator of the oxidative status of cells (Alía et al., 2005) being induced by *t*-BOOH when added to Caco-2 cells. Pre-treatment of cells with 10 μg/mL GPE and 1-10 μM GA or SA significantly reduced ROS over-production induced by *t*-BOOH. This result shows that the high levels of ROS generated were significantly quenched by the antioxidant compounds in cells pre-treated for 20 h. This ROS-quenching effect by GPE, GA and SA could be a first explanation for the reduced oxidative stress and subsequent cell protection.

GSH is a substrate in glutathione peroxidase-catalysed detoxification of organic peroxides, which repairs free radical induced damage through electron-

transfer reactions. A dramatic depletion of GSH intracellular levels was observed when 400 μM *t*-BOOH was added to Caco-2 cells. Pre-treatment with 1-10 μg/mL GPE, 1-10 μM GA or 10 μM SA partly prevented the depletion of GSH induced by *t*-BOOH. Other studies have reported similar results with a cocoa phenolic extract and its main flavanols in Caco-2 cells submitted to acrylamide-induced oxidative toxicity (Ramiro-Rodriguez et al., 2011). Moreover, it should be highlighted that the loss of cellular GSH seems to play an important role in apoptotic signaling. Flavanols have recently shown to provide parallel protection by enhancing the activity of a number of protective GSH dependent enzymes (Ramos et al., 2011), which are essential in cancer prevention (Rodriguez-Ramiro et al., 2011).

The enzymatic constituents of antioxidant defence system play a crucial role against oxidative stress; thus, the significant increase in the activity of GPx and GR observed after 3 h treatment with 400 μM *t*-BOOH clearly indicates a positive response in the cell defence system to face the intense ROS production in the presence of the prooxidant and overcome the oxidative insult (Alía et al., 2006). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will position the cell in a favorable condition to deal with a new challenge (Alía et al., 2006; Baeza et al., 2014; Sarriá et al., 2012). Accordingly, we have previously reported that realistic concentrations of cocoa (Martín et al., 2010) and green coffee (Baeza et al., 2014) extracts averted cell damage by preventing the permanently increased activity of GPx and GR induced by *t*-BOOH. The present study has shown that pre-treatment of cells with 1-10 μg/mL GPE and 1-10 μM GA or SA can efficiently return GPx and GR activities to basal values preparing cells to further oxidative insults. These results, together with those of GSH, indicated that the prevention or delay of appearance of conditions causing oxidative stress in the cell may also reflect the ability of a compound to modulate the cellular antioxidant defences.

Oxidative stress often leads to loss in specific protein or enzymatic function due to macromolecular damage. Carbonyl groups are considered as consistent biomarkers of oxidative damage to proteins, because of its relatively early formation and relative chemical stability of oxidised proteins and a crucial event in the development of cellular toxicity (Dalle-Donne et al., 2003). Pre-treatment of Caco-2 with 1-10 μg/mL GPE and 0.1-10 μM GA or SA decreased protein oxidation in response to the stressful situation. Chemo-protective effects on oxidative markers has also been reported with a cocoa phenolic extract and its

main flavonoids (Rodríguez-Ramiro et al., 2011) in the same cell line, and with derivatives from olive oil hydroxytyrosol (Pereira-Caro et al., 2012b) and green coffee phenolic compounds (Baeza et al., 2014) in a liver-derived cell line.

Attending to the outcome above, the smallest concentrations used in the present study did not show a significant protection against *t*-BOOH. These results are in agreement with Sun et al. (2014) who described no prevention of ROS generation or lipid peroxidation when cells were pre-incubated with 0.1 μM GA for 24 h before $Na_2S_2O_4$ exposure. GA is well absorbed in humans compared with other polyphenols and is found in plasma and urine, reaching concentrations up to 4 μM (Shahrzad and Bitsch, 1998; Lu et al., 2006). However, dietary phenolic compounds are limited by their absorption and metabolization rate (Manach et al., 2005; Deiana et al., 2012). *In vivo* experiments reported that GA is metabolized into methylation (unconjugated and conjugated 4-O-methylgallic acid, 2-O-methylgallic acid), decarboxylation (unconjugated and conjugated pyrogallol, 4-O-methylpyrogallol), and dehydroxylation (resorcinol) (Lu et al., 2006). Such high metabolism of hydroxybenzoic acids favours their disappearance as pure compounds from circulation and rapid excretion, and explains the need for doses in the micromolar range in order to effectively regulate antioxidant responses and exhibit chemo-protective capacity. Nevertheless, activity of phenolic compounds metabolized by intestinal microbiota should not be discarded since microbial-derived metabolites from cocoa flavonoids have shown anti-diabetic properties in cultured beta cells (Fernández-Millán et al., 2014).

Conclusions

GPE is a wine by-product rich in hydroxybenzoic acids, flavonols and hydroxycinnamic acids. Treatment of human Caco-2 cells in culture with physiological doses of GPE and its major phenolic compounds, GA and SA, provides cells with significant protection against toxicity by oxidative stress. This chemo-protection prepares Caco-2 cells and potentially enterocytes to face oxidative damage induced by dietary toxins. Further studies in animals and humans are needed to assess this biological activity of GPE and confirm its potential as a nutraceutical.

Chapter 2

In this chapter the protective effects of green coffee and yerba mate extracts, their main phenolic compound (5-caffeoylquinic acid) and derived microbial metabolites (dihydrocaffeic and dihydroferulic acids) against oxidative stress and inflammation induced by TNF-α was studied in human endothelial EA.hy 926 cells.

Study to be published as: Wang, S., Sarriá, B., Mateos, R., Goya, L., Bravo, L. TNF-α induced inflammation in human EA.hy926 endothelial cells is prevented by yerba mate and green coffee extracts, their main hydroxycinnamic acids, and microbial metabolites

Summary

Yerba mate has shown anti-inflammatory effects, whereas those in coffee are less clear. In previous studies, the main phenolic compound in mate and coffee, 5-caffeoylquinic acid (5-CQA) and their major metabolites originated by colonic microbiota, dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids have shown oxidative stress protective effects, particularly the former. In the present work, endothelial dysfunction was induced by TNF-α in human EA.hy926 cells and the protective effects of yerba mate (YME) and green coffee bean extracts (GCBE), 5-CQA, DHCA and DHFA at a wide range of concentrations, including physiological doses, against oxidative stress markers and on endothelial nitric oxide synthase (eNOS) activity were studied. Direct treatment with YME, GCBE, 5-CQA, DHCA and DHFA at the concentrations studied was not cytotoxic, and produced no or small positive changes. TNF-α treatment induced oxidative stress in EA.hy926 cells increasing the production of reactive oxygen species (ROS), inducing glutathione (GSH) depletion, increments in glutathione peroxidase (GPx) and reductase (GR) activities, and protein oxidation (determined as carbonyl groups, CG), also reducing eNOS levels. However, pre-treatment with YME, GCBE, 5-CQA, DHCA and, to a lower extent DHFA, at most of the concentrations assayed lowered ROS production, recovered depleted GSH, and reduced GR and GPx activities and carbonyl levels. Moreover, anti-inflammatory effects were observed as eNOS concentration was enhanced. In conclusion, physiological concentrations of 5-CQA, DHCA and DHFA may protect EA.hy926 cells against induced endothelial dysfunction.

Keywords: Yerba mate, green coffee bean, hydroxycinnamic acid metabolites, endothelial dysfunction, EA.hy926 cells, oxidative stress, TNF-α

Introduction

Yerba mate, *Ilex paraguariensis* St. Hil. (Aquifoliaceae) has been popular for centuries and is widely consumed by large populations in South America. Yerba mate has shown positive health effects related to its antioxidant, vaso-dilating, lipid-reducing, anti-glycation, anti-inflammatory and weight reducing properties, among others (Bracesco, et al. 2011). Many of these properties have been attributed to its rich content in phenolic compounds which amount up to 8.1-9.8 % of yerba mate dry weight (Bravo et al., 2007). Among these phenols, mate is particularly rich in caffeoyl derivatives (caffeic acid, 3-, 4-, and 5-caffeoylquinic acids (CQA), 3,4-, 3,5- and 4,5-dicaffeoylquinic acids (DCQA)), generically named as chlorogenic acids (CGAs), accounting for over 90% of the total phenolic content; in addition it contains flavonoids (quercetin, rutin and kaempferol glycosides) (Bravo et al., 2007; de Mejía et al., 2010).

Coffee is another beverage broadly consumed worldwide, which also presents a high content in phenolic compounds, mainly CGAs, that may amount up to 4.1–11.3% (w/w) of the green coffee bean (Perrone et al., 2010). The main phenolic compounds in green coffee beans are very similar to those in yerba mate: 3-, 4-, and 5-CQA, 3,4-, 3,5-, and 4,5-DCQA acids and 3-, 4-, and 5-feruloylquinic acids (FQA) (Alonso-Salces et al., 2009).

However, CGAs are poorly bioavailable and approximately 30% of the ingested dose is excreted in urine after extensive metabolism (Stalmach et al., 2009; Duarte and Farah, 2011; Renouf et al., 2014). Early metabolites appear in plasma plasma about 1–1.5 h after the intake of CGAs-rich beverages in μM-nM concentrations, mostly conjugated caffeic and ferulic acids, as well as caffeoylquinic acid metabolites, pointing to partial de-esterification of CGAs and metabolism by phase II conjugating enzymes after absorption in the small intestine (Monteiro et al., 2007; Farah et al., 2008; Renouf et al., 2010). However, major CGAs metabolites are dihydrocaffeic (DHCA) and dihydroferulic (DHFA) acids, which are generated by the action of the colonic microbiota, reaching μM concentrations in plasma 5–10 h after the intake of CGAs (Stalmach et al., 2009; Renouf et al., 2010, 2014; Gomez-Juaristi, 2015). These metabolites have a long half-life having been observed in urine 48 h after the intake of a single dose of coffee (Duarte and Farah, 2011).

The effects of coffee on inflammation are not clear, whilst there is certain evidence that mate has anti-inflammatory effects. In some human studies there were no effects of coffee on inflammation (Kempf et al., 2010; Gavrieli et al., 2011), whereas in others there was a tendency to reduce interleukine (IL)-6 and tumour necrosis factor (TNF)-α levels after sustained consumption for over a month (Yamashita et al., 2012). In contrast, coffee and coffee extracts have shown inhibitory effects on the expression of genes involved in inflammation (Paur et al., 2010), and 5-CQA and its metabolite caffeic acid seem to inhibit the expression of IL-1β and adhesion molecules soluble vascular cell adhesion molecule (sVCAM)- 1 and soluble intercellular cell adhesion molecule (sICAM)-1 (Chang et al., 2010). On the other hand, it seems that phenolic compounds in mate may inhibit nuclear translocation of the transcription factor NF-κB, suppressing the expression of pro-inflammatory cytokines in macrophages (Puangpraphant et al., 2011a). Mate has also shown to reduce metabolic and inflammatory markers in high-fat fed obese animals (Arcari et al., 2011; Borges et al., 2013; Pimentel et al., 2013; da Silva et al., 2014; Schinella et al., 2014), and in humans to inhibit plasma and lipoprotein oxidation and reduce plasma levels of oxidative stress biomarkers (da Silva et al., 2008; Boaventura et al., 2012). However, most of the cell and rat studies assessing the antioxidant and anti-inflammatory activity of CGAs have been performed using pure hydroxycinnamic acids, mainly 5-CQA, or the plant phenolic extracts. Since the major plasma metabolites after the intake of yerba mate and green coffee are DHCA and DHFA (Gomez-Juaristi, 2015), it is relevant to understand the antioxidant and anti-inflammation activity of these compounds compared to their parent molecules.

Cardiovascular diseases (CVD) are responsible for the highest rate of death around the world. One of the major complications in CVD is a chronic inflammation process based on the stiffness of the arteries, atherosclerosis and endothelial dysfunction. Nitric oxide (NO), generated in the endothelial cells from L-arginina by means of the enzyme endothelial NO synthase (eNOS), is the main factor responsible for vasodilation and maintenance of vascular tone, among other antiatherogenic effects (Andriantsitohaina et al. 2012). Vascular cells produce reactive oxygen species that may contribute to the dysfunction associated with CVD (Dhalla et al., 2000). The human umbilical cord endothelial EA.hy 926 cell line is an adequate model to study vascular complications in which there is an increase in oxidative stress and inflammation. The cell line has been

widely applied in the study of leukocyte adhesion to endothelial cells, oxidative stress and protein expression (Karbacha et al., 2012). Polyphenols may reduce endothelial cell vulnerability to oxidative stress at both the membrane and cytosol level (Barringer et al 2011; Carluccio et al., 2003) and stimulate eNOS through the activation of the oestrogen receptor-α of endothelial cells via the inhibition of the p38MAPK and PI3K/Akt pathways (Anter et al., 2005; Kane et al., 2009). By increasing NO levels, protection against oxidative stress, inhibition of angiogenesis, platelet activation and cell migration/proliferation are generated (Murphy et al., 2003; Pignatelli et al., 2006; Schewe et al., 2008; Holt et al., 2009; Vita, 2005). Polyphenols have also shown anti-inflammatory and immuno-modulatory effects attributed to direct antioxidant action or effects mediated by cell-signalling pathways like NF-κB and others (Schubert et al., 2007). Considering all the above, it is relevant to further investigate the protective effects of the main derivative microbial metabolites (DHCA and DHFA) of green coffee and yerba mate and their main phenolic compound (5-CQA), versus the extracts against oxidative stress and inflammation induced by TNF-α in EA.hy 926 cells.

Results

Viability, cytotoxicity and antioxidant effects of YME, GCBE, CQA, DHCA and DHFA on EA.hy926 cells in basal conditions

Table 15 shows the viability and cytotoxicity results obtained in basal conditions after direct treatment of EA.hy926 cells with YME, GCE (1, 20 and 50 µg/mL), CQA (0.1, 1, 10, 20 µM), DHCA and DHFA (0.1, 0.3, 1, 10 µM) for 24 h using the crystal violet and LDH assays, respectively. No statistically significant changes were found in cell viability, indicating that the concentrations selected for the study did not damage cell integrity during the period of incubation. Increase of LDH in culture medium may be considered a necrosis marker, i.e. high LDH values indicate a cytotoxic effect. Considering that the percentage of LDH after cells were incubated with the indicated concentrations of YME and GCBE, 5-CQA, DHCA and DHFA were similar to control cells, it may be concluded that no cytotoxic effects were observed. Moreover, Figure 27 (A-E) shows that EA.hy926 cells exposed to YME at 50 µg/mL, GCBE at 20 and 50 µg/mL, 5-CQA and DHCA at all the concentrations studied (0.1, 1, 10 and 20 µM) and DHFA at 0.3, 1 and 10 µM after 16 h incubation up to 24 hours showed a significant decrease

in the production of reactive oxygen species (ROS) compared to the control. Only YME at 50 µg/mL significantly increased ROS generation.

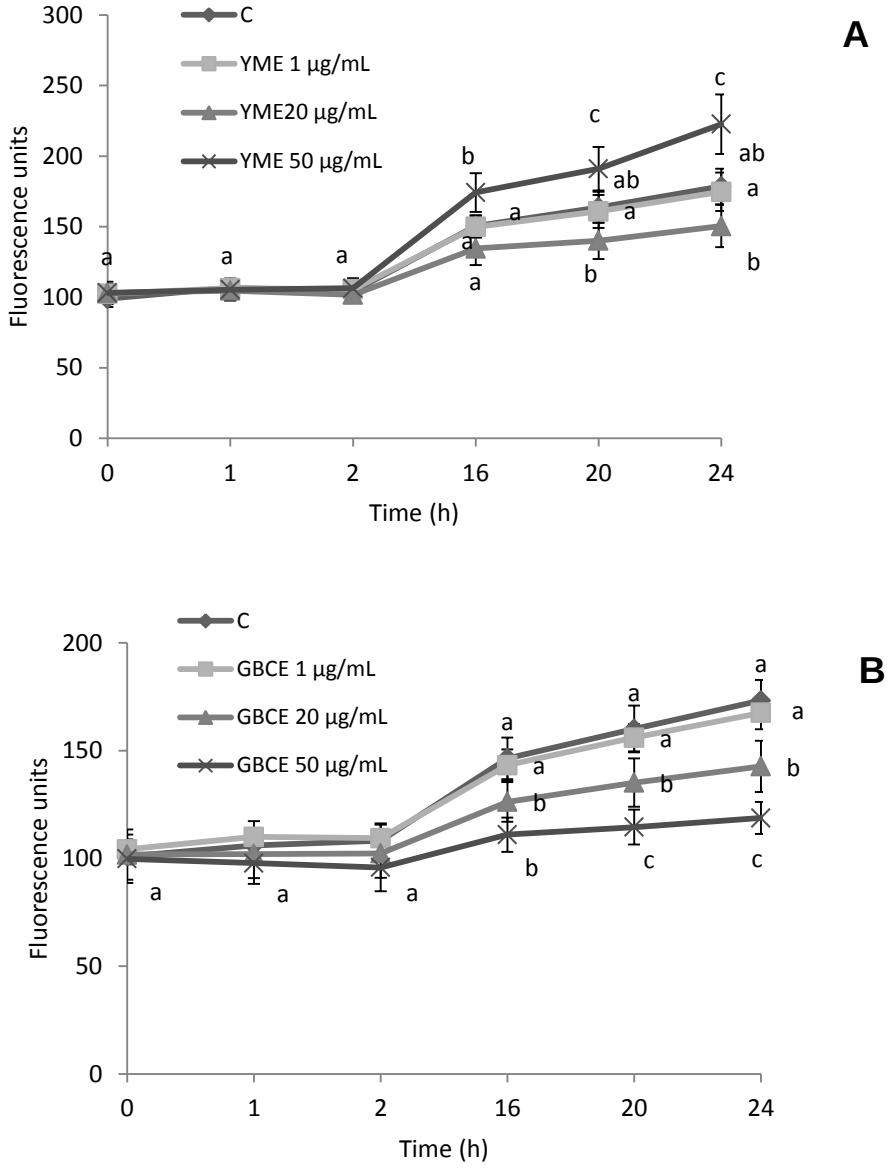


Figure 27. Direct effect on EA hy926 intracellular ROS generation after treatment with YME (A), GCBE (B), 5-CQA (C), DHCA (D) and DHFA (E). Results are expressed as fluorescence units against time (n=4–6). Different letters denote statistically significant differences within the same time period (p<0.05).

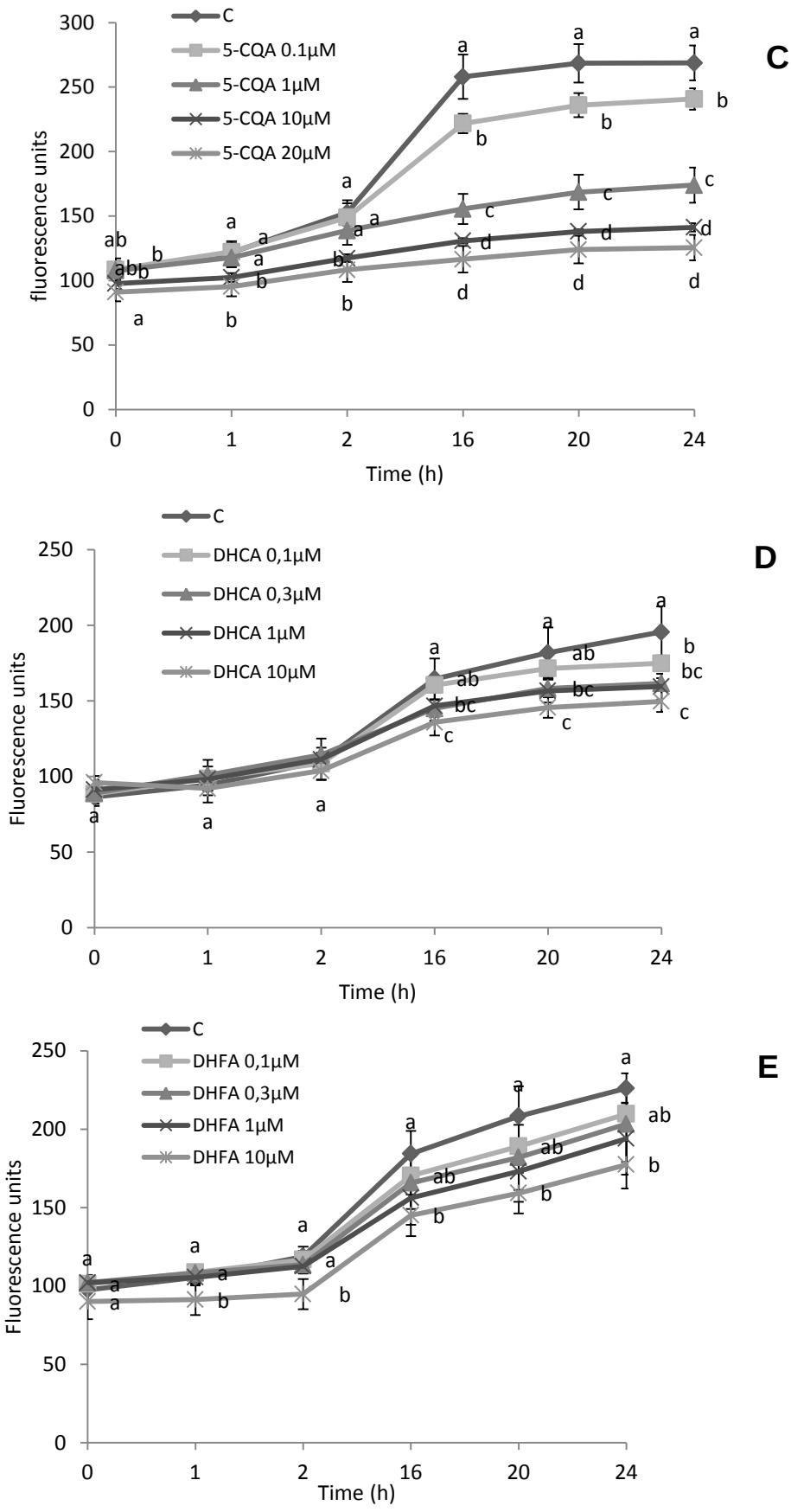


Figure 27 (Cont.). Direct effect on EA hy926 intracellular ROS generation.

Table 15. Direct effect of YME, GCBE, 5-CQA, DHCA and DHFA on EA hy926 cell viability, antioxidant defenses (GSH levels, and GPx and GR activities), protein damage (carbonyl groups, CG) and eNOS.

	Cell viability	LDH	GSH	GPx	GR	CG	eNOS
	%	%	ng/mg.prot	mU/mg.prot		nmol/mg prot	pg/mg.prot
Control	100.0±5.7[a]	17.4±0.9[a]	63.1±10.1[a]	154.23±9.75[a]	15.79±1.11[a]	1.5±0.19[a]	1,395.3±103.8[a]
YME (µg/mL)							
1	111.3±4.8[a]	19.0±2.1[a]	65.3±8.4[a]	155.7±12.7[a]	18.4±0.4[a]	1.7±0.3[a]	1,553.8±80.2[a]
20	106.7±8.8[a]	17.7±1.9[a]	72.7±4.5[a]	157.8±4.4[a]	15.8±2.7[a]	1.6±0.2[a]	1,425.2±128.8[a]
50	108.8±10.3[a]	17.4±1.7[a]	61.2±6.2[a]	135.4±15.7[a]	17.9±1.9[a]	1.6±0.1[a]	1,544.4±109.2[a]
GCBE (µg/mL)							
1	98.2±9.1[a]	17.3±1.5[a]	61.7±7.7[a]	156.4±14.5[a]	16.2±1.5[a]	1.8±0.2[a]	1,620.3±133.9[ab]
20	96.9±11.6[a]	17.3±2.2[a]	63.2±9.8[a]	147.2±10.5[a]	16.9±1.8[a]	1.5±0.2[a]	1,727.4±147.9[b]
50	99.6±4.4[a]	18.0±1.1[a]	68.2±9.2[a]	146.0±11.7[a]	16.2±1.6[a]	1.5±0.2[a]	1,689.8±149.0[b]
5-CQA (µM)							
0.1	109.7±6.4[a]	20.0±1.2[a]	63.9±7.2[a]	145.9±16.9[a]	19.1±1.1[a]	1.7±0.2[a]	2,127.6±133.9[a]
1	110.2±5.8[a]	19.1±1.8[a]	62.2±4.5[a]	142.4±9.6[a]	19.0±1.1[a]	1.6±0.3[a]	2,086.1±147.9[a]
10	106.4±8.1[a]	19.8±1.5[a]	62.1±3.2[a]	140.7±9.4[a]	19.2±2.6[a]	1.7±0.2[a]	2,033.2±169.0[a]
20	106.6±7.2[a]	18.2±1.9[a]	62.1±5.3[a]	150.7±9.6[a]	16.8±2.5[a]	1.5±0.2[a]	2,269.9±173.5[a]
DHCA (µM)							
0.1	104.5±11.1[a]	18.5±1.9[a]	62.0±5.7[a]	162.7±13.1[a]	17.5±1.4[a]	1.7±0.2[a]	1,976.8±73.2[a]
0.3	101.7±10.4[a]	18.9±1.1[a]	60.5±6.2[a]	160.6±10.1[a]	16.0±2.4[a]	1.6±0.2[a]	1,938.7±108.0[a]
1	100.8±6.4[a]	19.1±1.1[a]	60.1±2.6[a]	143.8±19.7[a]	17.0±1.3[a]	1.6±0.2[a]	1,929.0±80.6[a]
10	103.5±7.5[a]	17.3±1.4[a]	65.2±7.1[a]	139.8±12.5[a]	18.8±2.3[a]	1.5±0.2[a]	1,931.5±135.7[a]
DHFA (µM)							
0.1	101.4±11.0[a]	17.3±1.8[a]	65.1±9.0[a]	160.6±18.4[a]	18.3±2.6[a]	1.8±0.2[a]	2,096.3±124.1[a]
0.3	102.5±5.5[a]	19.0±1.1[a]	71.6±5.5[a]	153.1±13.8[a]	18.4±2.6[a]	1.8±0.1[a]	2,199.1±212.8[a]
1	98.7±5.5[a]	16.9±1.0[a]	66.5±4.2[a]	150.4±17.2[a]	16.1±0.7[a]	1.7±0.2[a]	2,244.5±198.9[a]
10	106.3±8.4[a]	19.0±1.9[a]	66.7±9.4[a]	141.7±4.4[a]	18.7±2.4[a]	1.6±0.2[a]	2,233.1±211.9[a]

Results expressed as mean ± SD (n = 4-8). Different letters within a column denote statistically significant differences ($p<0.05$).

With respect to the antioxidant defence system, there were no statistically significant changes in glutathione (GSH) levels either in the activity of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR). Accordingly, there were no statistical differences in protein oxidation after 20 h treatment with the extracts, phenolic compounds and metabolites as shown by the levels of carbonyl groups (Table 15). Thus, it can be assumed that the range of concentration tested can be safely used to study the protective effect of YME, GCBE, 5-CQA, DHCA and DHFA against a condition of oxidative stress in the endothelial cells. Accordingly, after treatment with the different compounds and extracts for 20 h, eNOS protein levels were not altered, except for GCBE which caused a statistically significant increase at 20 µg/mL.

Protective effects of YME, GCBE, 5-CQA, DHCA and DHFA in EA.hy926 cells in a condition of oxidative stress and inflammation

In order to study the protective effect of pre-treatment of EA.hy926 cells with YME and GCBE, 5-CQA, DHCA, and DHFA for 20 h against the oxidative stress and inflammation induced by TNF-α, firstly, the cells were treated with 5, 10, 20 and 40 ng/mL of TNF-α for 24 h and the absence of cytotoxicity was verified by the crystal violet method (Figure 28A). The concentrations tested were selected attending to the literature: 5 ng/mL TNF-α in 3T3-L1 pre-adipocytes (Yen el al., 2011), 10 ng/mL TNF-α in in HUVEC cells (Wang et al., 2014), EA.hy936 cells (Jia et al., 2015; Yang et al., 2014), Caco-2 cells (Rodriguez-Ramiro et al., 2013), and in HBMEC cells (Lee et al., 2008), and 20 and 40 ng/mL TNF-α in Caco-2 cells (Rodriguez-Ramiro et al., 2013). Afterwards, another experiment was carried out in EA.hy926 cells to measure the effects of 5, 10, 20 and 40 ng/mL TNF-α for 0.5, 1, 2, 4, 20, 24 h on ROS production (Figure 28B). After 20 h treatment, 5, 10, 20 and 40 ng/mL TNF-α induced a sharp increase in ROS production, which was even higher at 24 h. Attending to the results of these experiments, TNF-α at 10 ng/mL for 24 h was the condition selected to induce oxidative stress in the present work and to evaluate the protective effects of YME, GCBE, 5-CQA, DHCA, and DHFA in EA.hy926 cells against this condition. In these experiments, a negative control, cells non-treated with TNF-α, and a positive control, cells treated with TNF-α at 10 ng/mL for 24 h but not exposed to the extracts, phenolic compounds or metabolites, were used.

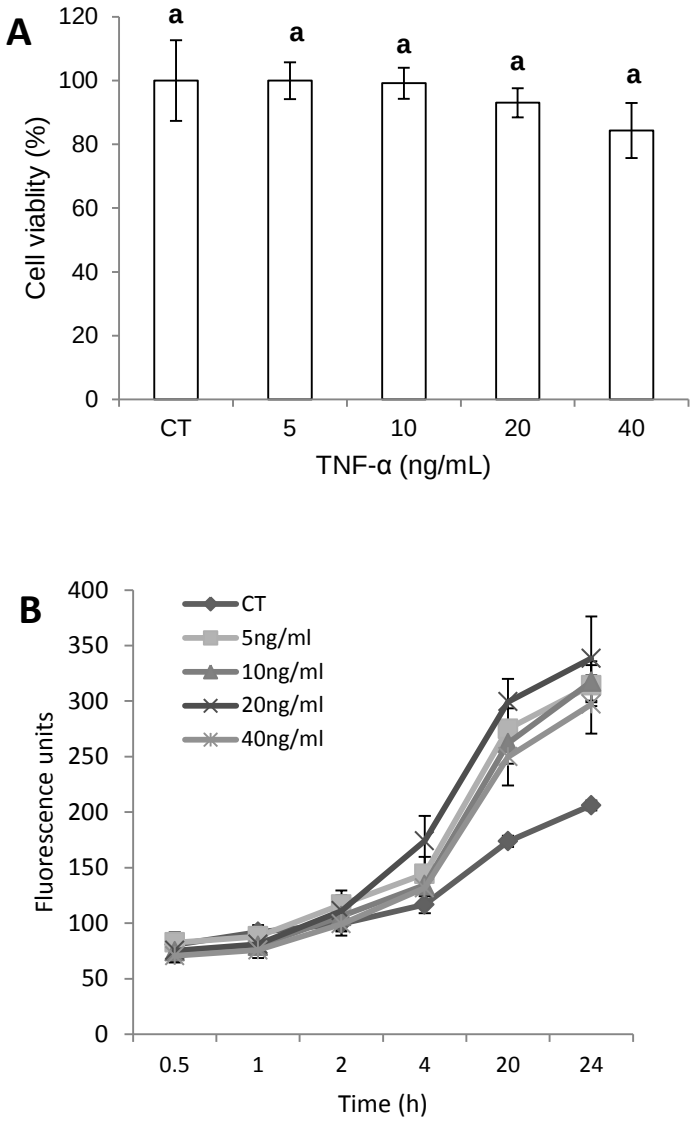


Figure 28. Effect of TNF-α on cell viability and ROS production in EA.hy926 cells. Values are means and the vertical bars are standard deviations. (A) Cells were treated with 5, 10, 20 and 40 ng/mL TNF-α for 24 h and cell viability was measured. (B) Cells were treated with 5, 10, 20 and 40 ng/mL TNF-α for 0.5, 1, 2, 4, 20, 24 h and ROS production was measured.

Similar experiments to the above described were carried out in EA.hy926 cells in order to evaluate the effects of different concentrations of TNF-α and incubation times on the concentration of eNOS. Figure 29A shows that TNF-α significantly decreased eNOS concentration from the concentration of 10 ng/mL to 40 ng/mL compared to control cells, whereas Figure 29B shows that after exposing EA.hy926 cells to 10 ng/mL of TNF-α at different incubation times, a

reduction of eNOS takes place from 4 till 24 hours compared to the control, showing the lowest levels after 24 h. Attending to these outcomes, the treatment conditions were considered adequate to evaluate the possible protective effects of the extracts, phenolic compound and metabolites on endothelial nitric oxide synthase (eNOS).

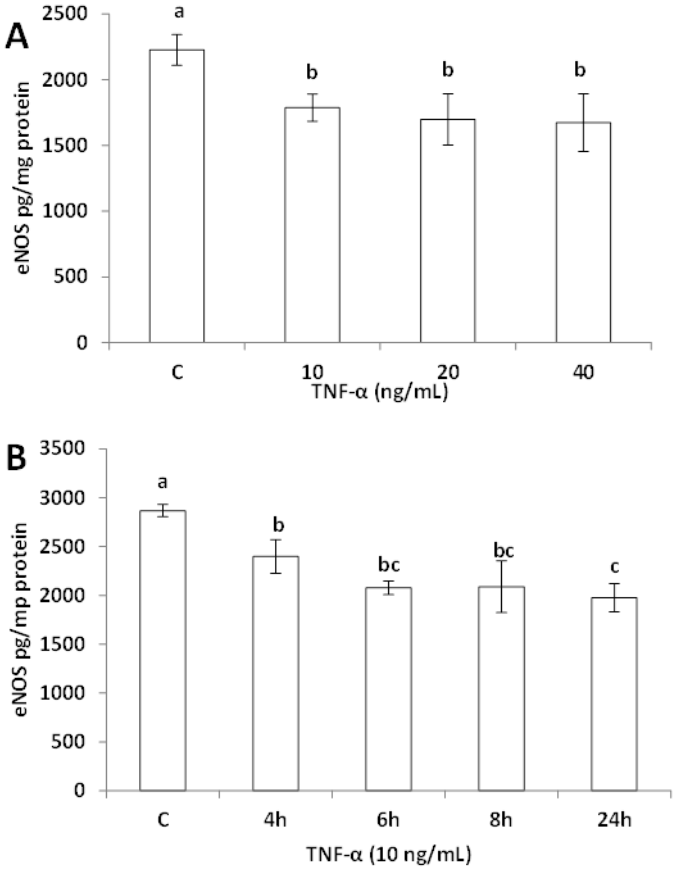


Figure 29. Effect of TNF-α on eNOS in EA hy926 cells. Values are means with standard deviations represented by vertical bars. (A) Cells were treated with 10, 20, and 40 ng/mL TNF-α for 24 h. (B) Cells were treated with 10 ng/mL TNF-α for 4, 6, 8, and 24 h.

Cell cytotoxicity

Treatment with TNF-α at 10 ng/mL after 24 h significantly enhanced (1.4 times) LDH leakage compared with the negative control, indicating prominent cell damage in EA.hy926 cells (Figure 30). However, when the cells were pre-treated with YME and GCBE at 20 and 50 µg/mL, a significant decrease in the

cytotoxicity occurred, whereas with 1 µg/mL of both extracts the decrease was small and not statistically relevant. However, 5-CQA at all the concentrations tested (0.1, 1, 10, and 20 µM) and DHCA at 0.3, 1, and 10 µM significantly decreased the cytotoxicity induced by TNF-α compared with the stressed control, in contrast to DHFA that did not reduce LDH levels (Figure 30).

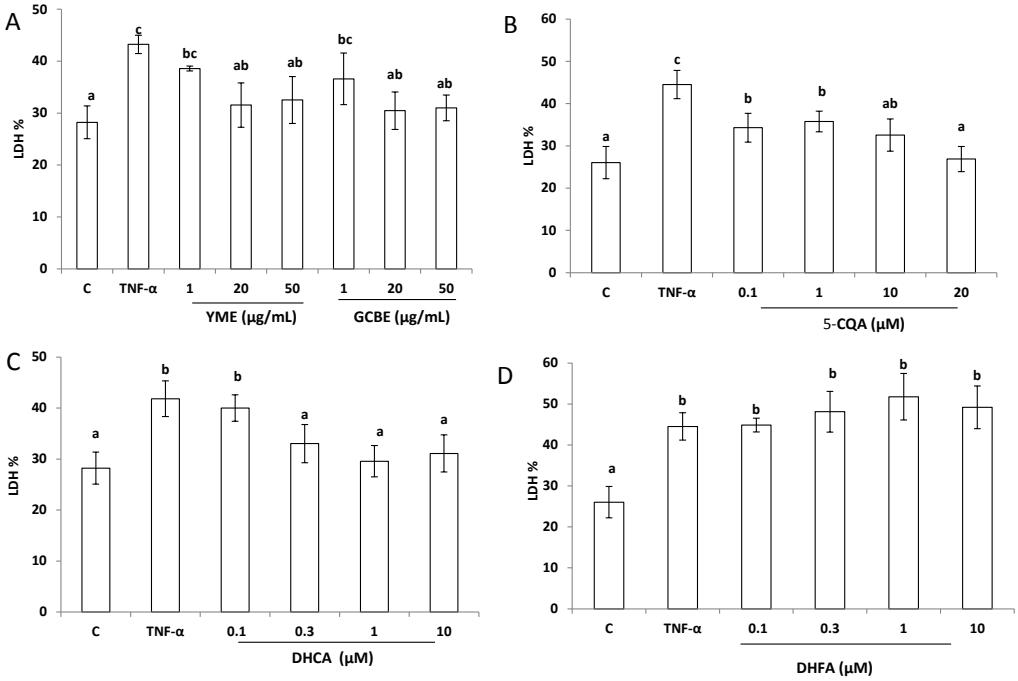


Figure 30. Protective effect of pre-treatment with YME and GCBE (A), 5-CQA (B), DHCA (C) and DHFA (D) for 20 h on cell toxicity (%LDH) induced by TNF-α for 24 h. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences (p<0.05).

ROS generation

EA.hy926 cells treated with the pro-inflammatory molecule for 24 h showed an approximate 40% increase in intracellular ROS generation compared to non-stressed controls (Figure 31). Pre-treatment with YME and GCBE at 20 and 50 µg/mL, 5-CQA at 1, 10, and 20 µM, and DHCA at 0.3, 1, and 10 µM significantly decreased ROS production compared to the TNF-α control, i.e. the lower concentrations of test compounds did not induce significant reductions.

Similarly to in the previous experiment and Baeza et al. (2016), DHFA failed to protect the cells form the pro-oxidant effect of TNF-α.

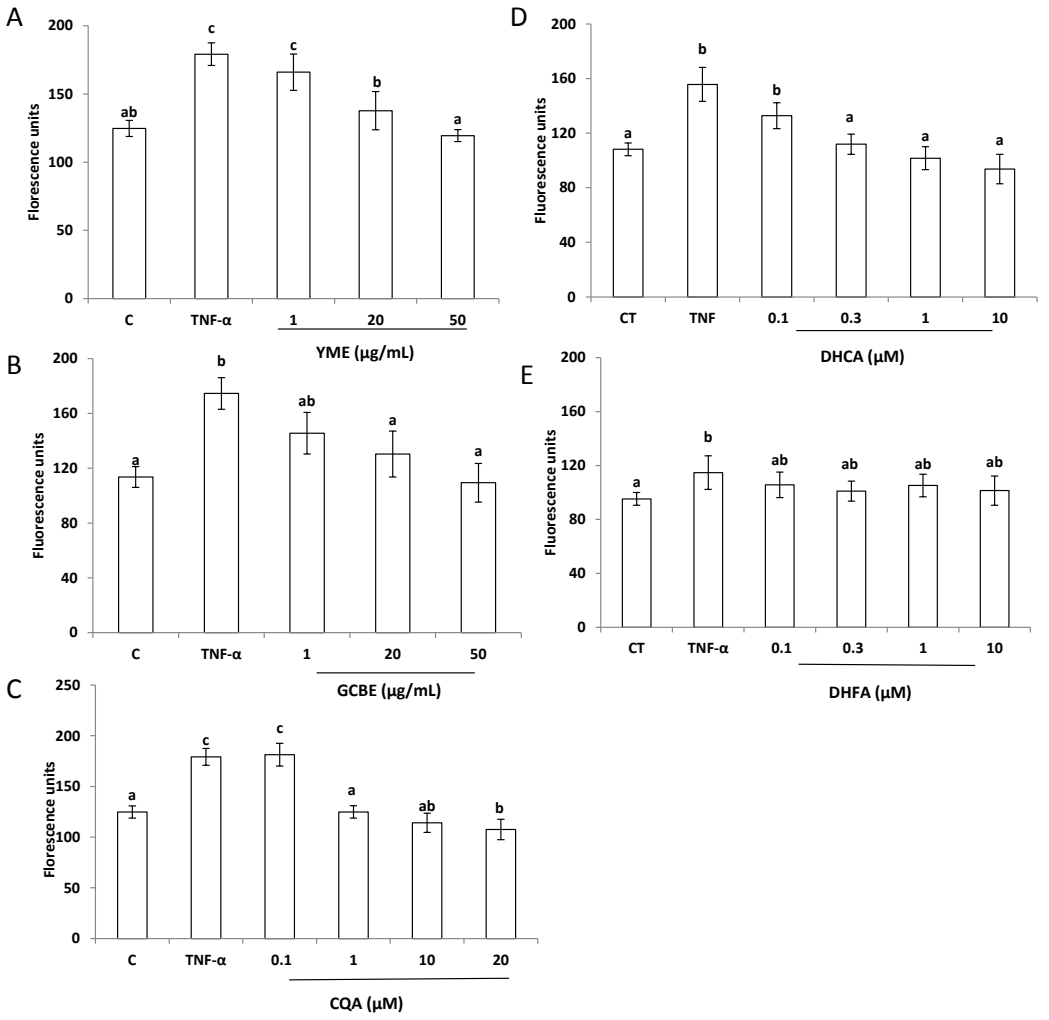


Figure 31. Protective effect on EA hy926 cell intracellular ROS production pre-treated with the noted concentrations of YME (A), GCBE (B), 5-CQA (C), DHCA (D) and DHFA (E), and after TNF-α exposure for 60 min. Results are expressed as % of fluorescence arbitrary units against time (n = 4–6). Different letters within the same time denote statistically significant differences (p<0.05).

Antioxidant defences

Non-enzymatic defences: TNF-α in EA.hy926 cells evoked around a 30% decrease in the steady-state concentration of GSH (Figure 32). Pre-treatment with 20 and 50 µg/mL YME, 50 µg/mL GCBE, 1-20 µM 5-CQA, and 1-10 µM DHCA significantly increased GSH levels, partly recovering the TNF-α-induced

depleted concentrations. DHFA only afforded a partial recovery of GSH levels at the highest (10 µM) dose tested (Figure 32D).

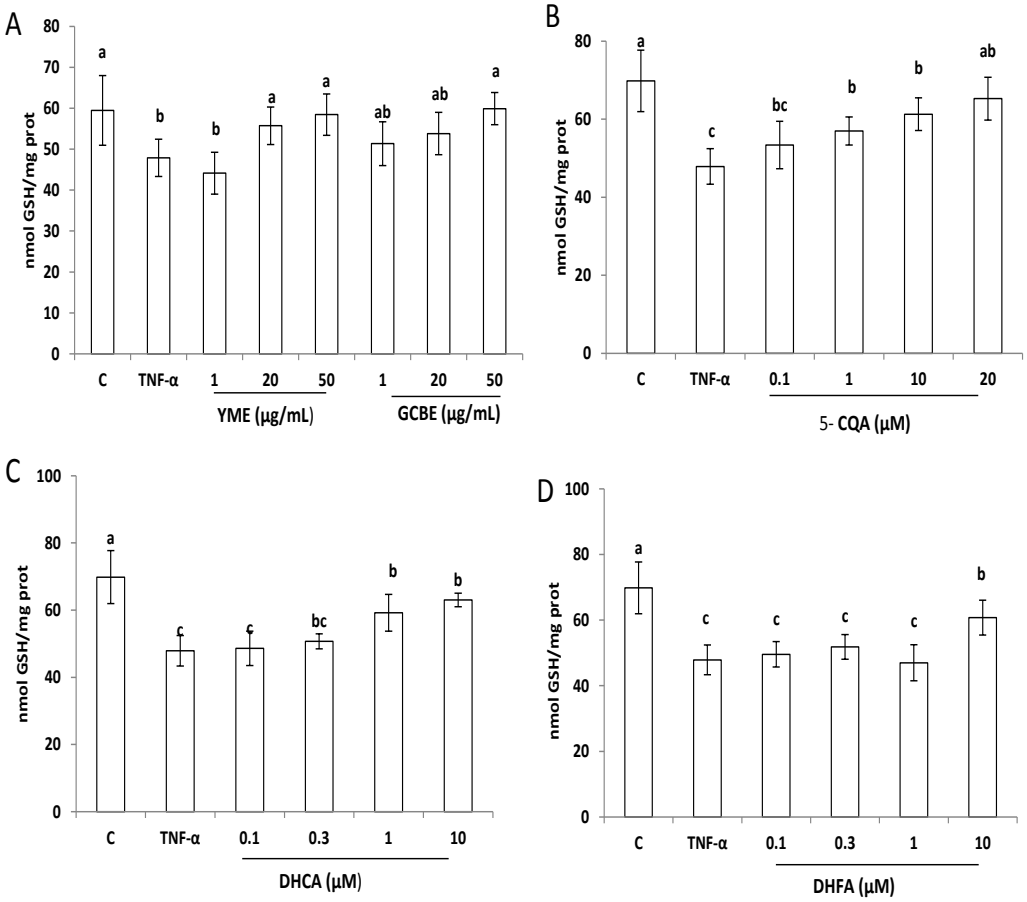


Figure 32. Protective effect of pre-treatment with YME, GCBE (A), 5-CQA (B), DHCA (C) and DHFA (D) for 20 h, and after TNF-α exposure for 24 h on GSH levels in EA.hy926 cells. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences (p<0.05).

Enzymatic defences: The enzyme activities of GPx were about 150% increased after incubation with 10 ng/mL TNF-α in EA.hy926 cells, indicating a positive response of the cell defence system to the pro-inflammatory challenge (Figure 33). When cells were pre-treated with 20-50 µg/mL YME and GCBE, 10-20 µM 5-CQA, and 10 µM DHCA for 20 h, the chemically-induced rise in GPx activity was prevented compared with stressed cells, whilst DHFA had no effect.

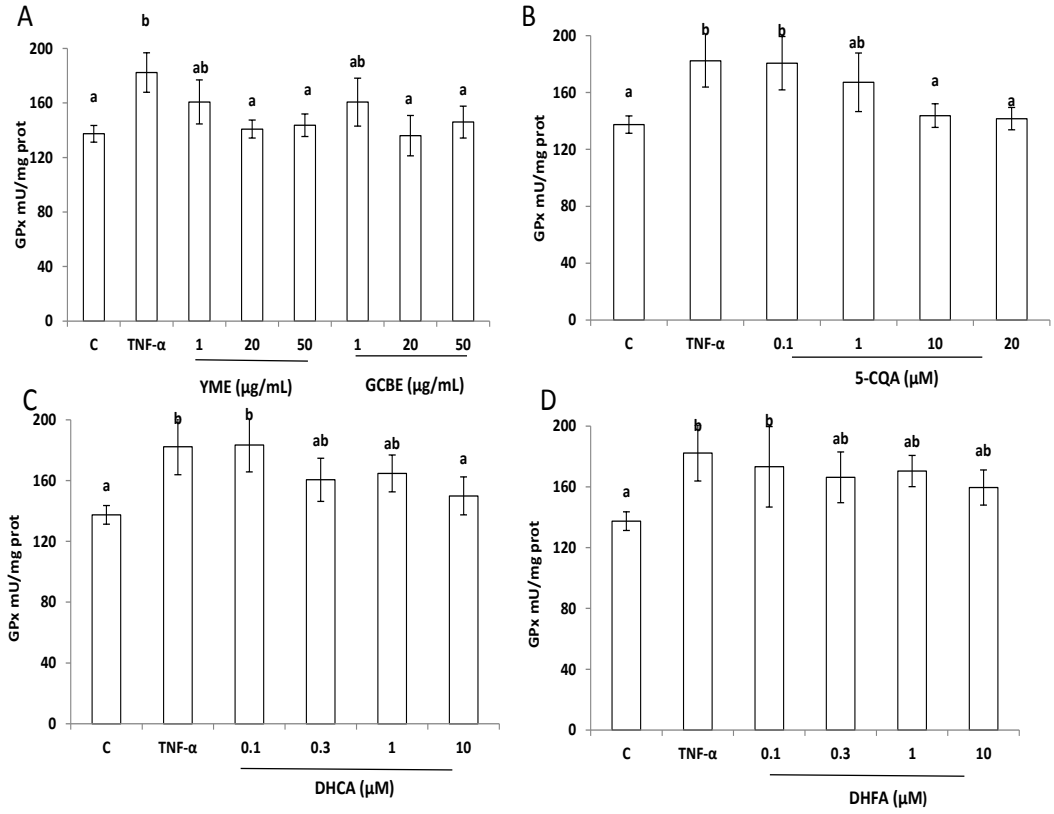


Figure 33. Protective effect of pre-treatment with YME, GCBE (A), 5-CQA (B), DHCA (C) and DHFA (D) for 20 h, and after TNF-α oxidative insult for 24 h on EA.hy926 cell antioxidant enzyme GPx. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences (p<0.05).

In line with GPx results, GR over-activation was also restricted compared with TNF-α treated cells after pre-incubation of EA.hy926 endothelial cells with 1-50 µg/mL YME, 20-50 µg/mL GCBE, 1-20 µM 5-CQA, and 0.3-10 µM DHCA, while only the highest dose (10 µM) of DHFA showed some effect on GR activity (Figure 34). Lower concentrations of YME, GCBE, 5-CQA, and DHCA did not significantly decrease GPx and GR activities (Figures 33 and 34).

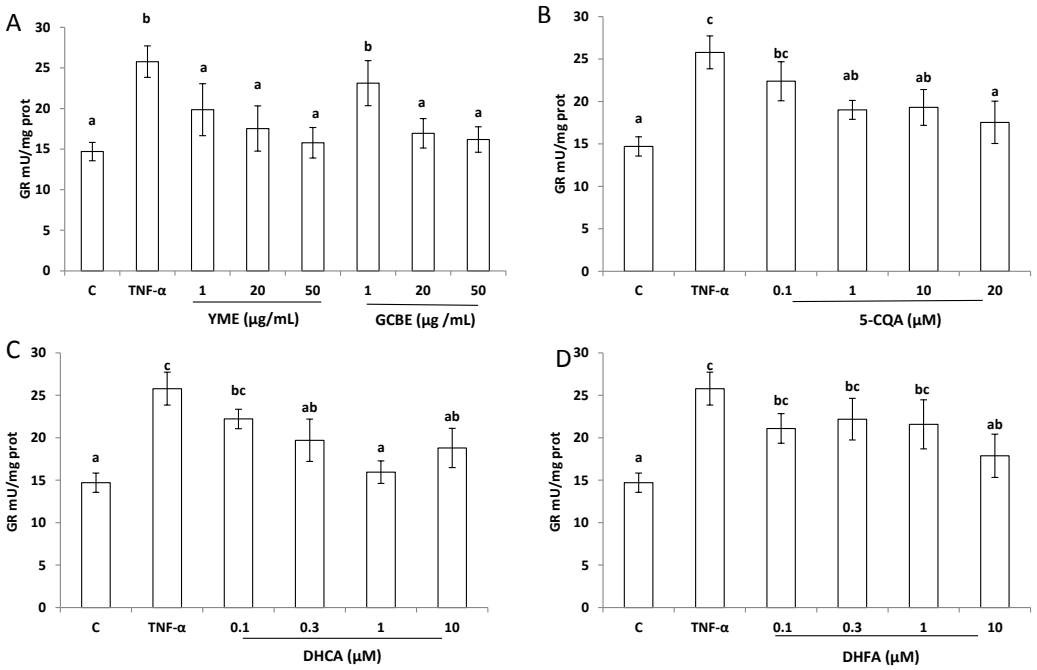


Figure 34. Protective effect of pre-treatment with YME, GCBE (A), 5-CQA (B), DHCA (C) and DHFA (D) for 20 h, and after TNF-α oxidative insult for 24 h on EA.hy926 cell antioxidant enzyme GR. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences ($p<0.05$).

Biomarker of protein oxidative damage

Endothelial EA.hy926 cells treated with 10 ng/mLTNF-α for 24 h showed a significant increase in the cellular concentration of carbonyl groups, indicating oxidative damage to cell proteins (Figure 35). However, pre-treatment of EA.hy926 cells with 1-50 µg/mL YME and GCBE, 1-20 µM 5-CQA, and 0.1-10 µM DHCA and DHFA for 20 h significantly reduced the level of carbonyl groups in response to TNF-α, indicating a reduced level of protein oxidation in response to the stressor.

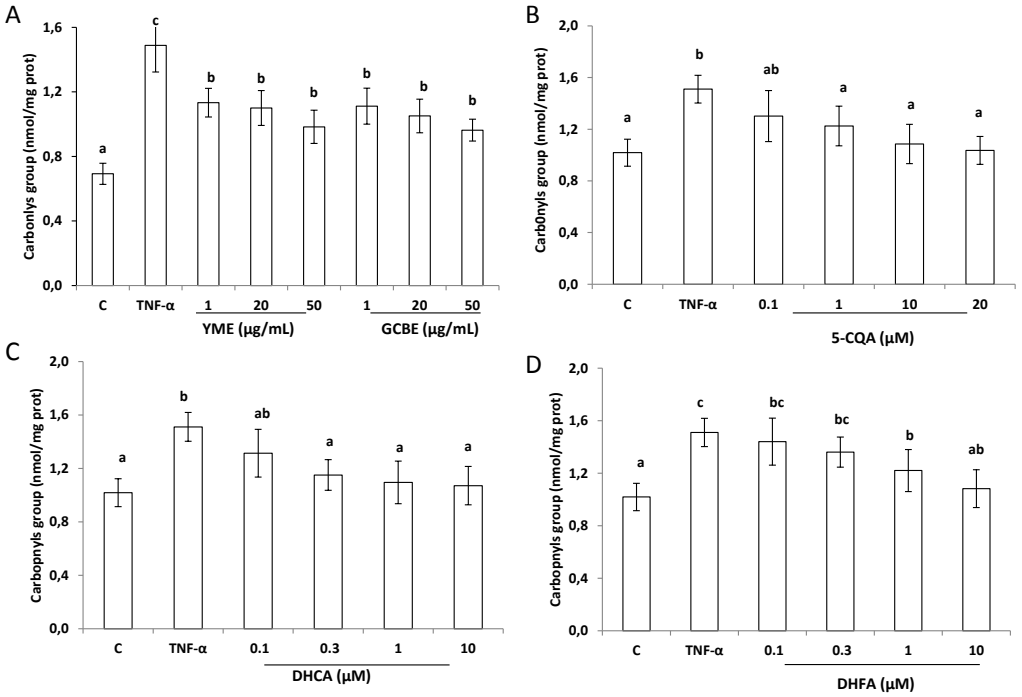


Figure 35. Protective effect of pre-treatment with YME, GCBE (A), 5-CQA (B), DHCA (C) and DHFA (D) against oxidative stress induced by TNF-α for 24 h on the EA.hy926 cell protein-oxidation (carbonyl groups). Results are means ± SD (n=4–8). Different letters denote statistically significant differences (p<0.05).

The concentration of endothelial nitric oxide synthase (eNOS)

The effects of the treatment with YME, GCBE, 5-CQA, DHCA and DHFA, at the indicated concentrations on the levels of endothelial nitric oxide synthase were also investigated in TNF-α stimulated EA.hy926 cells.

Treatments with YME, GCBE and 5-CQA increased eNOS concentration in a dose dependent manner. In contrast, DHCA and DHFA did not show a dose-dependent response, increasing eNOS levels similarly at all the concentrations tested, even the lowest 0.1 µM dose (Figure 36).

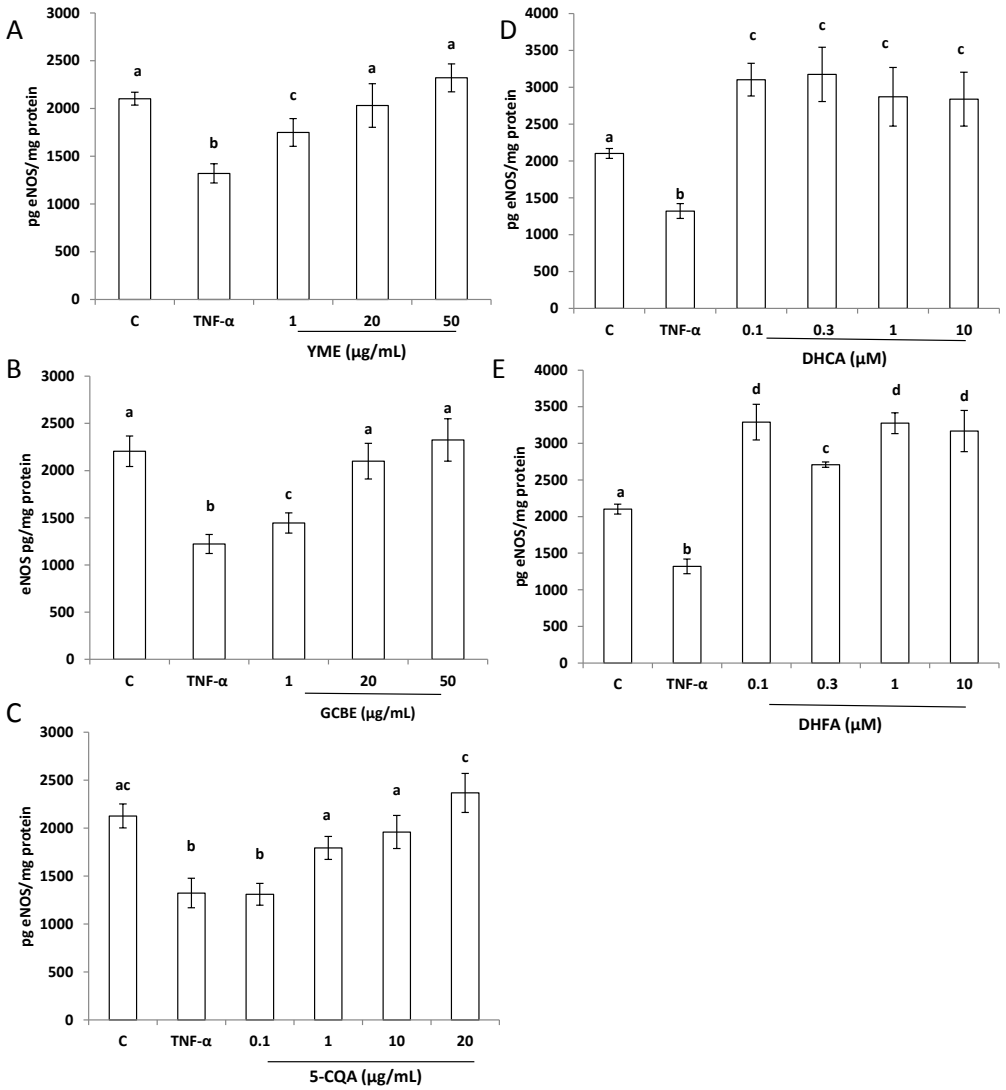


Figure 36. Protective effect of pre-treatment with YME (A), GCBE (B), 5-CQA (C), DHCA (D) and DHFA (E) against oxidative stress induced by TNF-α for 24 h on the eNOS levels in EA.hy926 cells. Results are means ± SD (n=4–8). Different letters denote statistically significant differences (p<0.05).

Discussion

The consumption of yerba mate and coffee, mainly due to their rich content in CGAs, has been reported to reduce the risk of human chronic diseases such as cardiovascular disease (van Dam and Feskens, 2002; Ding et al., 2014; de Moraes Portilho et al., 2015). However, the bioavailability of CGAs is largely dependent on its metabolism by microorganisms in the gut (Olthof et al., 2001; Nardini et al., 2002; Gonthier et al., 2003), so that maximum plasma

concentration of DHFA and DHCA have been observed 8 h after coffee consumption (Lang et al., 2013). In two studies recently carried out in our group, a yerba mate and green bean phenolic extract showed high capacity to protect human HepG2 cells against oxidative stress inducted by *tert*-butylhydroperoxide (*t*-BOOH) being the protective effect of the microbial metabolites DHFA and DHCA, particularly that of DHCA, even at low, physiological concentrations (Baeza et al., 2016). In the other study, also 5-CQA and 3,5-DCQA, presented significant protection against oxidative stress in the same cell model (Baeza et al., 2014), in agreement with other research groups that described antioxidant effects of 5-CQA in PC-12 cells (Park, 2013).

Before investigating the chemo-protective effects of the extracts, 5-CQA, DHCA and DHFA, it was crucial to evaluate their cellular safety at the concentrations tested. Of these, it was particularly relevant to test the effects of DHCA and DHFA at 0.1 and 0.3 µM as these concentrations have been observed in human bioavailability studies carried out in our group using yerba mate and green/roasted coffee infusions (Gómez-Juaristi, 2015). None of the doses of YME, GCBE, 5-CQA, DHCA and DHFA induced direct cell damage or cytotoxic effects (Table 15). YME at 1 and 20 µg/mL, GCBE at 20 and 50 µg/mL, 5-CQA at 0.1, 1, 10 and 20 µM (Figure 27 A-E), DHCA at 1 and 10 µM, and DHFA at 10 µM significantly reduced ROS generation. In contrast, YME at 50 µg/mL showed pro-oxidant effects. Moreover, antioxidant defences such as GSH, GPx and GR were not altered by any treatment as the levels of carbonyl groups as biomarkers of protein oxidation (Table 15). Therefore, all the concentrations studied of the extracts, phenolic compound and metabolites, except YME at 50 µg/mL, were favourable conditions for the endothelial cells to face an oxidative stress challenge; in addition, these compounds enhanced the production of eNOS, although not reaching the level of statistical significance, except GCBE at 20 and 50 µg/mL.

TNF-α induced clear oxidative stress conditions in the vascular cells used, in agreement with previous studies (Chen et al., 2009; Granado-Serrano et al., 2012). Before starting the study, different TNF-α concentrations and incubation times were tested to set up an adequate oxidative stress model using EA.hy926 endothelial cells. TNF-α at concentrations above 5 ng/mL induced a significant increase in ROS production after 2 h incubation and reached even higher levels after 24 h (Figure 28), without inducing cell death (measured by the crystal violet

assay) even at the highest concentration (40 ng/mL). ROS production is a good indicator of the oxidative status of cells (Alia et al., 2005), therefore, we selected to use TNF-α at 10 ng/mL, which is above the healthy physiological range (14·2–61·7 pg/mL) according to Kokkonen et al. (2010), which are doses already applied by other authors (Granado-Serrano et al., 2012; Rodríguez-Ramiro et al., 2013), using an exposure time of 24 h to assure an oxidative stress condition.

Cells treated with TNF-α also showed high LDH leakage. However, pre-treatment with YME, GCBE, 5-CQA, and DHCA exhibited a significant decrease in cytotoxicity, except for DHFA which showed no effect (Figure 30). Similarly, TNF-α-induced increase in ROS generation was efficiently quenched in EA.hy926 cells pre-treated with the assayed extracts and phenolic compound. The metabolite DHCA was as effective as the parent compound (5-CQA) decreasing ROS generation in TNF-α-challenged cells, whilst DHFA showed lower efficiency, with all the tested doses (0.1-10 μM) slightly reducing ROS levels although not reaching statistical significance.

Regarding GSH levels, it is usually assumed that GSH depletion reflects intracellular oxidation, whereas a balanced GSH concentration is expected to prepare the cells against a potential oxidative insult (Alia et al., 2006b). In the present study, treatment with TNF-α induced a significant decrease in intracellular GSH levels; however, pre-treatment with YME and GCBE extracts, 5-CQA, and DHCA partly prevented the depletion of GSH, in agreement with other studies using apocyanin and tempol (Mariappan et al., 2007), or a cocoa phenolic extract and its main phenolic compounds (epicatechin and procyanidin B2) (Rodríguez-Ramiro et al., 2011b).

TNF-α also dramatically affected antioxidant enzymes in EA hy 926 cells, evoking a significant increase of GPx and GR activities to cope with ROS increase and thus pointing to a positive response of the endothelial cell defence system to face the oxidative insult. However, cells pre-treated with YME, GCBE, 5-CQA, DHCA, and the highest dose of DHFA reduced antioxidant enzyme activities returning to control values. Similar protective effects have also been described for cocoa phenolic (Martin et al., 2013), green coffee beans (Baeza et al., 2014) and grape pomace (Wang et al., 2016).

Carbonyl groups are considered a consistent biomarker of oxidative damage to proteins, because of its relatively early formation and the chemical

stability of oxidised proteins (Dalle-Donne et al., 2003). Pre-treatment with all the tested compounds significantly reduced the levels of carbonyl groups, similarly to other phenolic compounds: epicatechin (Martin et al., 2010a), quercetin (Alia et al., 2006b), hydroxytyrosol (Goya et al., 2007) in HepG2 cells, and cocoa epicatechin in the pancreatic beta cell line INS-1E (Martin et al., 2014).

As aforementioned, eNOS is the main factor responsible for vasodilation and maintenance of vascular tone, among other anti-atherogenic effects (Andriantsitohaina et al., 2012). To different degrees, a red wine phenolic extract (Leikert, 2002), cyanidin, *p*-coumaric acid, caffeic acid, benzoic acid, and vanillic acid (Wallerath et al., 2005) have shown to enhance eNOS expression, as well as resveratrol metabolites (Ladurner et al., 2014). Conversely, quercetin produced null or even negative effects on eNOS expression (Huisman et al., 2004; Tribolo et al., 2013). However, to our knowledge, this is the first study that evaluates the effects of YME, GCBE, CGA, DHCA and DHFA on eNOS activity in endothelial cells. We observed a dose-dependent increase with both extracts, 5-CQA, DHCA, and DHFA. Regarding the metabolites, it is noteworthy that there was a significant increase in eNOS levels from the lowest concentrations, pointing to these metabolites having potent anti-inflammatory properties. In all, it is a very interesting finding that both microbial metabolites have clear antioxidant and anti-inflammation properties. Between the two, DHCA showed higher bioactivity, its effect starting at lower concentrations in several of the markers studied. The difference between both molecules is the methylation of the hydroxyl group of the phenolic ring, which suggests the importance of the ortho-diphenolic group in the biological activity of polyphenols (Bravo, 1998; Huang et al., 2005).

Conclusion

The pro-inflammatory cytokine TNF-α induced oxidative stress and inflammatory damage in EA.hy926 endothelial cells and may be used as an *in vitro* model to study biological effects of food components at this level. Comparing the two extracts, yerba mate showed stronger antioxidant effects than GCBE, whereas 5-CQA also showed clear antioxidant and anti-inflammatory effects. Physiological concentrations of microbial metabolites (DHCA and DHFA) can prevent oxidation acting at different levels (attenuating ROS production, through enzymatic and non-enzymatic antioxidant enzymes, preventing protein

oxidation) and in addition may improve eNOS, thus providing endothelial protection. The molecular mechanisms involved in the endothelial protection observed deserved to be further studied.

Chapter 3

The aim of the present study was to explore the association between coffee and yerba mate consumption and cancer. The effect of phenolic extracts of these beverages and their major phenolic compounds, metabolites and methylxanthines was studied in normal cells and in four (colon, lung, oesophagus and urinary bladder) human cancer cell lines. Results show that yerba mate extracts and to a lower degree green coffee extracts and hydroxycinnamic acids decrease viability and proliferation of cancer cells.

Studied will be published as: Amigo-Benavent, M., Wang, S**.,** Mateos, R., Sarriá B, Bravo, L. Antiproliferative and cytotoxic effects of green coffee and yerba mate extracts, their main hydroxycinnamic acids, methylxanthines, and microbial metabolites in human Caco-2, A549, OE-33, and T24 cancer cell lines.

Antiproliferative and cytotoxic effects of green coffee and yerba mate extracts and their main bioactive compounds and metabolites in human cancer cell lines

Abstract

Consumption of green coffee and yerba mate as beverages or dietary supplements is increasing due to their health benefits. This work aimed at studying the effects of polyphenol-rich extracts of green coffee beans (GCBE) and yerba mate (YME), and the main phenolic components (5-caffeoylquinic acid, 5-CQA; 3,5-dicaffeoylquinic acid, 3,5-DCQA) and metabolites (ferulic acid, FA; caffeic acid, CA; dihydrocaffeic acid, DHCA; and dihydroferulic acid, DHFA) along with caffeine (CAF) on the viability and proliferation of human cancer cell lines. Different concentrations of extracts (10-1000 μg/mL) and standards (10-1000 μM) were assayed in colon adenocarcinoma (Caco-2), lung (A549), oesophageal (OE-33), and urinary bladder (T24) human carcinoma cells, as well as in a non-cancer colon cell line (CCD-18Co). Cells were treated for 2 and 24 h, mimicking acute and chronic exposure, respectively. YME significantly reduced cell viability in all the cancer cell lines at all the concentrations tested, the higher doses also reducing cell proliferation. GCBE effects on cell viability were more effective at 100 and 1000 μM, showing modest effects on cell proliferation, except in Caco-2 and T24. 5-CQA and 3,5-DCQA reduced cell viability and proliferation in all cell lines at the higher concentrations studied, whereas FA, DHCA and DHFA presented lower, variable effects depending on the cell line and the concentration used. Caffeine had no effect in any of the cell lines. Since the physiologically relevant dose of 10 μg/mL YME showed remarkable effects decreasing cell viability and proliferation, lower concentrations (0.1, 1 and 10 μg/mL) were tested for cytotoxicity and effects on reactive oxygen species (ROS) generation. These parameters were also determined in CCD-18Co cells with YME, and 5-CQA, CA and DHCA (0.1, 1 and 10 μM), since these compounds affected this non-cancer cell line. However, low concentrations had no cytotoxic effect, even YME being cytoprotective in some cells and all tested compounds in CCD-18Co. In conclusion, yerba mate and to a lower degree green coffee extracts, and their phenolic components and metabolites may decrease the viability and proliferation of cancer cells.

Introduction

The consumption of green coffee and yerba mate as dietary supplements or beverages is increasing due to their reported health benefits, since they have been associated with lower risk of suffering diseases of oxidative aetiology (Kozuma et al., 2005; Nkondjock, 2009; Esquivel and Jimenez, 2012). Numerous studies have evaluated the association between coffee consumption and the risk of cancer in humans (Bøhn et al., 2014). Certain results indicate coffee reduces the risk of colon (Giovanucci, 1998), endometrial (Shimazu et al., 2009), liver, kidney, bladder, and breast cancers (Nkondjock, 2009), whereas other reports suggest that coffee consumption increases bladder (Kurahashi et al., 2009), breast, lung (Tang et al., 2009; Tang et al., 2010), and laryngeal cancers (Chen and Long, 2014). In contrast, other studies did not relate coffee to increased incidence of prostate, breast (Bhoo-Pathy et al., 2015), pancreas or ovary cancers (Nkondjock, 2009; Turati et al., 2012).

The relationship between yerba mate and cancer has been much less studied compared with coffee. Mate has shown antimutagenic effects in cell culture and animal models (Nkondjock, 2009; Heckman et al., 2010; Bracesco et al., 2011). However, epidemiological studies have described a link between yerba mate consumption and oral cavity, pharynx, oesophagus, and oropharyngeal cancers (Goldenberg, 2002; 2003, Dasanayake et al., 2010). This has been associated to the high temperature mate is traditionally consumed in South America, adding boiling water to mate leaves in a “calabaza” recipient and drinking the hot brew through a metallic straw-like implement called “bombilla”. Consumption of mate tea with bombilla has also been associated with bladder cancer in ever-smokers, but not in never-smokers, whereas “mate cocido” (leaves infused in hot water, consumed at lower temperatures) was not associated with this type of cancer (Bates et al., 2007). There is no sound population-based, case-control study on mate consumption that establishes yerba mate as a risk factor for cancer (Loria et al., 2009)

Cell culture studies have shown an antiproliferative effect of polyphenols in mate against oral cancer cells, inhibiting topoisomerases I and II (de Mejia et al., 2005), in addition to showing an increase in apoptosis and necrosis in human lymphocytes (Wnuk et al., 2009) or a protective effect against DNA damage induced by H_2O_2 in liver, kidney and bladder cells isolated from mice (Miranda et

al., 2008). This effect was also observed in Caco-2 cells after incubation with chlorogenic acid, an extract of green coffee or bread supplemented with antioxidants from green coffee (Glei et al., 2006). The cell viability reduction and induction of apoptosis in tumor cells after treatment with hydroxycinnamic acids (Miccadei et al., 2008) was related to their antioxidant activity, increasing the activity and expression of antioxidant enzymes in lung and skin tumor cells, suppressing cell signals involved in survival and proliferation (NF-κB, AP-1 and MAPK) (Feng et al., 2005), although in another cell model (human hepatoma cells) such effects were not observed (Granado-Serrano et al., 2007).

Hydroxycinnamic acids are the main phenolic compounds present in yerba mate and green coffee, which may amount up to 8.10-9.77% of the yerba mate dry weight (Bravo et al., 2007) and to 4.1-11.3% (w/w) of the coffee bean (Baeza et al., 2014). Major hydroxycinnamic acids described in these products are 3-, 4-, and 5-caffeoylquinic acids (3, 4-, and 5-CQA), 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids (3,4, 3,5-, and 4,5-DCQA), and 3-, 4-, and 5-feruloylquinic acids (3-, 4-, and 5-FQA), among others (Alonso-Salces et al., 2009). Yerba mate and green coffee have also in common a high content of the trimethylxanthine caffeine. The bioavailability and biotransformation of hydroxycinnamic acids and caffeine have been studied in human hepatoma HepG2 cells (Mateos et al., 2006), rats (Fernandez-Gomez et al., 2016) and in humans (Stalmach et al., 2009; Martínez-López et al., 2014; Gomez-Juaristi, 2015). These compounds are absorbed, metabolised and modified by the colonic microbiota. Derived CQAs, FQAs and caffeine have been reported to reach maximum plasma concentrations of 143 nM, 158 nM and 10.50 μM, respectively, with urine concentrations of 11.1 μM, 40.3 μM and 4.12 μM, respectively (Stalmach et al., 2009; Martínez-López et al., 2014). Dihydrocaffeic acid (DHCA) and dihydroferulic acid (DHFA) are major microbial metabolites found in plasma and urine derived from CQAs and FQAs. Maximum concentration of DHFA and DHCA in plasma of 0.878 μM and 0.678 μM have been described 8 h after coffee consumption, respectively (Lang et al., 2013).

Free caffeic (CA) and ferulic (FA) acids can be minor components in coffee and mate, although they are more commonly found esterified to quinic acid, glycosylated, etc. (Baeza et al., 2016). However, they are also formed after absorption of the corresponding parent molecules due to the action of phase I metabolizing enzymes (i.e. esterases, glycosidases), and further metabolized by

phase II enzymes (sulphated, glucoronidated, methylated, etc.). Plasma concentrations of metabolites in the nM range have been determined after the ingestion of coffee (45 nM) and yerba mate (65 nM) (Gomez-Juaristi, 2015).

The aforementioned discrepancies on the effect of coffee and yerba mate on cancer may be due to the bioactive components originally present in the beverages and/or to the molecules that are formed after being absorbed, metabolised and modified by the colonic microbiota. Therefore, to further understand the association between coffee and yerba mate with cancer, the aim of this work was to study the effects of phenolic extracts of coffee and yerba mate, their main compounds and resulting metabolites on the viability and proliferation of colon (Caco-2), lung (A549), oesophagus (OE33) and urinary bladder (T24) cancer cell lines.

Results

Cells were exposed for 2 and 24 h to a range of concentrations of GCBE and YME (10-1000 μg/mL) and pure compounds (10-1000 μM of caffeine, 5-CQA, 3,5-DCQA, CA, FA, DHCA and DHFA) as described in Materials and Methods.

Effects of extracts, polyphenols and metabolites on cell viability and proliferation

YME reduced cell viability to a higher degree than GCBE (Table 15). Treatment for 2 and 24 h with YME induced a patent negative dose-dependent effect on cell viability in all the studied cell lines. GCBE also decreased cell viability, although contrary to YME, the lowest concentration (10 μg/mL) only showed differences with respect to the control after 24 h of treatment in OE-33 and T24 cancer cells. Even 100 μg/mL GCBE showed modest effects and only the highest dose (1000 μg/mL) affected cell viability with reductions between 87% (CCD-18Co, 2h) to only 22% (Caco-2, 24 h) with respect to control cells (Table 15).

Table 15. Effect of green coffee bean and yerba mate extracts on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33 and T24) human cell lines.

Cell lines	Treatment (µg/mL)	Green coffee bean extract				Yerba mate extract			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100±7 ^{a}	100±3 ^{a}	100±12 ^{a}	100±11 ^{a}	100±7 ^{a}	100±3 ^{a}	100±12 ^{a}	100±11 ^{a}
	10	96±5 ^{a}	95±5 ^{a}	108±11 ^{a}	95±9 ^{a}	37±2 ^{b}	49±4 ^{b}	99±9 ^{a}	88±12 ^{b}
	100	64±2 ^{b}	82±5 ^{b}	98±9 ^{a}	78±10 ^{b}	6±1 ^{c}	14±1 ^{c}	96±10 ^{a}	53±4 ^{c}
	1000	13±1 ^{c}	22±2 ^{c}	101±9 ^{a}	66±6 ^{c}	2±1 ^{d}	6±1 ^{d}	1±0 ^{b}	1±1 ^{d}
Caco-2	C	100±10 ^{a}	100±4 ^{ab}	100±5 ^{a}	100±6 ^{a}	100±10 ^{a}	100±4 ^{a}	100±5 ^{a}	100±6 ^{a}
	10	102±8 ^{a}	105±5 ^{a}	92±4 ^{b}	103±10 ^{a}	77±7 ^{b}	89±6 ^{b}	85±5 ^{b}	99±6 ^{a}
	100	93±6 ^{a}	95±7 ^{b}	86±4 ^{bc}	98±10 ^{a}	33±5 ^{c}	62±9 ^{c}	82±6 ^{b}	73±7 ^{b}
	1000	52±3 ^{b}	78±7 ^{c}	80±5 ^{c}	79±8 ^{b}	4±1 ^{d}	0±0 ^{d}	25±2 ^{c}	22±2 ^{c}
A549	C	100±4 ^{ab}	100±5 ^{a}	100±9 ^{ab}	100±8 ^{a}	100±4 ^{a}	100±5 ^{a}	100±9 ^{a}	100±8 ^{a}
	10	103±4 ^{a}	98±5 ^{a}	94±4 ^{a}	95±7 ^{a}	84±3 ^{b}	73±5 ^{b}	101±9 ^{a}	76±6 ^{b}
	100	95±5 ^{b}	89±6 ^{b}	108±9 ^{b}	80±8 ^{b}	39±3 ^{c}	41±2 ^{c}	91±8 ^{a}	58±5 ^{c}
	1000	59±6 ^{c}	55±4 ^{c}	104±5 ^{ab}	70±7 ^{b}	29±4 ^{d}	2±0 ^{d}	20±1 ^{b}	17±2 ^{d}
OE-33	C	100±4 ^{a}	100±6 ^{a}	100±5 ^{a}	100±7 ^{a}	100±4 ^{a}	100±6 ^{a}	100±5 ^{a}	100±10 ^{a}
	10	95±3 ^{a}	85±8 ^{b}	106±10 ^{a}	112±7 ^{a}	52±3 ^{b}	55±4 ^{b}	98±10 ^{ab}	96±8 ^{a}
	100	70±5 ^{b}	74±7 ^{c}	99±9 ^{a}	109±10 ^{a}	16±1 ^{c}	27±2 ^{c}	85±8 ^{b}	65±5 ^{b}
	1000	23±3 ^{c}	27±3 ^{d}	99±8 ^{a}	103±8 ^{a}	12±1 ^{d}	15±2 ^{d}	15±2 ^{c}	16±0 ^{c}
T24	C	100±5 ^{a}	100±9 ^{a}	100±7 ^{a}	100±6 ^{a}	100±5 ^{a}	100±9 ^{a}	100±7 ^{a}	100±6 ^{a}
	10	99±4 ^{a}	78±3 ^{b}	105±5 ^{a}	102±9 ^{a}	74±5 ^{b}	88±8 ^{b}	92±9 ^{a}	61±4 ^{b}
	100	84±3 ^{b}	78±7 ^{b}	83±9 ^{b}	103±8 ^{a}	31±3 ^{c}	70±7 ^{c}	71±7 ^{b}	14±3 ^{c}
	1000	39±3 ^{c}	61±6 ^{c}	61±4 ^{c}	9±3 ^{b}	10±1 ^{d}	9±1 ^{d}	2±1 ^{c}	1±1 ^{d}

Values are expressed as the mean ± SD. Different letters within a column and cell line indicate significant differences ($p<0.05$).

While cell viability determines the capacity of cells to survive in the presence of the tested extracts or compounds, cell proliferation provides evidences of the capacity of the cells to grow and multiply, which is of especial relevance in cancer cells with enhanced proliferation capacity. Short treatment with GCBE did not modify the proliferation capacity of A549 and OE-33 cancer cells, although it decreased proliferation in Caco-2 and bladder T24 cells (in the latter only the highest concentrations). At longer incubation time (24 h), only the highest levels of GCBE were able to reduce proliferation of all but OE-33 cells, also affecting non-cancerous CCD-18Co cells (Table 15).

As for YME, the highest concentration (1000 µg/mL) was able to significantly reduce cell proliferation in all cell lines after 2 h, totally blocking proliferation of CCD-18Co and T24 cells. The 100 µg/mL dose also decreased proliferation of OE-33 and T24 cells, while Caco-2 cells, as with GCBE, were affected for all YME concentrations after 2 h. Treatment with YME for 24 h dose-dependently decreased cell proliferation in all cell lines (except Caco-2 and OE-33, where the 10 µg/mL dose was not effective) (Table 15).

Treatment for 2 and 24 h with 100 and 1000 µM 5-CQA dose-dependently reduced cell viability in both non-cancer and cancer cell lines (Table 16). The 10 µM dose, however, only affected viability of OE-33 cells (and CCD-18Co) at 2 h incubation time, surprisingly increasing the viability of A549 cells. This, however, was not accompanied by an increased proliferation, since only the 1000 µM dose of 5-CQA was able to decrease proliferation of CCD-18Co and T24 cells at short incubation times, also decreasing proliferation of all cell lines after 24 h treatment, except A549, which again showed a different response with higher proliferation after 24 h with 100 µM 5-CQA (Table 16).

As with the monocaffeoylquinic acid, 3,5-DCQA dose-dependently reduced viability of all cell lines after 2 and 24 h incubation, but only with the highest concentrations. This compound showed some effects decreasing cell proliferation of cancer cells at 2 and 24 h treatment with the highest doses. Only Caco-2 and T24 cells showed a slight yet statistically significant reduction of cell proliferation after 24 h treatment with 10 µM 3,5-DCQA (Table 16).

Table 16. Effect of 5-caffeoylquinic and 3,5-dicaffeoylquinic acids on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33 and T24) human cell lines.

Cell lines	Treatment (μM)	5-caffeoylquinic acid				3,5-dicaffeoylquinic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100±5 ^{a}	100±7 ^{a}	100±10 ^{a}	100±10 ^{a}	100±8 ^{a}	100±7 ^{a}	100±12 ^{a}	100±11 ^{a}
	10	83±4 ^{b}	93±5 ^{ab}	105±8 ^{a}	100±10 ^{a}	102±5 ^{a}	98±7 ^{a}	100±9 ^{a}	94±9 ^{a}
	100	64±3 ^{c}	88±6 ^{b}	95±7 ^{a}	91±8 ^{a}	88±6 ^{b}	86±6 ^{b}	103±9 ^{a}	71±8 ^{b}
	1000	39±2 ^{d}	48±4 ^{c}	80±6 ^{b}	75±9 ^{b}	34±2 ^{c}	1±2 ^{c}	95±8 ^{a}	14±2 ^{c}
Caco2	C	100±5 ^{a}	100±7 ^{a}	100±8 ^{a}	100±8 ^{a}	100±4 ^{ab}	100±9 ^{a}	100±8 ^{a}	100±6 ^{a}
	10	102±7 ^{a}	105±7 ^{a}	96±9 ^{a}	110±10 ^{a}	108±6 ^{a}	102±7 ^{a}	97±10 ^{a}	89±4 ^{b}
	100	86±6 ^{b}	81±4 ^{b}	100±10 ^{a}	107±6 ^{a}	97±6 ^{b}	97±8 ^{a}	86±6 ^{a}	64±5 ^{c}
	1000	43±4 ^{c}	30±2 ^{c}	91±8 ^{a}	77±6 ^{b}	73±5 ^{c}	78±5 ^{b}	75±6 ^{b}	68±5 ^{c}
A549	C	100±6 ^{a}	100±7 ^{a}	100±8 ^{a}	100±8 ^{a}	100±2 ^{a}	100±7 ^{a}	100±10 ^{a}	100±8 ^{a}
	10	116±8 ^{b}	97±5 ^{a}	89±6 ^{a}	107±11 ^{ab}	101±8 ^{a}	92±6 ^{ab}	89±8 ^{a}	97±10 ^{ab}
	100	94±7 ^{a}	79±6 ^{b}	92±7 ^{a}	120±10 ^{b}	84±6 ^{b}	82±7 ^{b}	69±5 ^{b}	86±6 ^{bc}
	1000	63±4 ^{c}	51±4 ^{c}	90±7 ^{a}	107±10 ^{ab}	47±4 ^{c}	38±3 ^{c}	69±7 ^{b}	74±6 ^{c}
OE-33	C	100±6 ^{a}	100±8 ^{a}	100±7 ^{a}	100±10 ^{a}	100±3 ^{a}	100±3 ^{a}	100±5 ^{a}	100±10 ^{a}
	10	87±3 ^{b}	80±7 ^{b}	106±10 ^{a}	90±3 ^{ab}	102±3 ^{a}	96±5 ^{a}	100±7 ^{a}	96±8 ^{a}
	100	72±3 ^{c}	68±4 ^{c}	98±10 ^{a}	84±6 ^{b}	75±4 ^{b}	73±3 ^{b}	96±9 ^{ab}	98±8 ^{a}
	1000	30±3 ^{d}	15±1 ^{d}	91±9 ^{a}	61±9 ^{c}	21±2 ^{c}	5±0 ^{c}	87±8 ^{b}	18±2 ^{b}
T24	C	100±4 ^{a}	100±5 ^{a}	100±5 ^{a}	100±10 ^{a}	100±7 ^{a}	100±5 ^{a}	100±7 ^{a}	100±6 ^{a}
	10	94±4 ^{a}	99±4 ^{a}	100±10 ^{a}	103±8 ^{a}	102±10 ^{a}	95±6 ^{a}	92±6 ^{a}	90±6 ^{b}
	100	88±4 ^{b}	96±5 ^{a}	105±11 ^{a}	110±9 ^{a}	82±7 ^{b}	84±7 ^{b}	92±7 ^{a}	48±6 ^{c}
	1000	21±2 ^{c}	29±4 ^{b}	75±8 ^{b}	64±4 ^{b}	45±4 ^{c}	0±0 ^{c}	20±2 ^{b}	3±2 ^{d}

Values are expressed as the mean ± SD. Different letters within a column and cell line indicate significant differences ($p<0.05$).

Table 17. Effect of caffeic and ferulic acids on the cell viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33 and T24) human cells.

Cell lines	Treatment (μM)	Caffeic acid				Ferulic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100±6 [a]	100±7 [a]	100±8 [a]	100±8 [a]	100±6 [a]	100±7 [a]	100±8 [a]	100±8 [a]
	10	79±5 [b]	96±6 [a]	96±10 [a]	99±6 [a]	97±8 [a]	99±8 [a]	109±9 [a]	103±5 [a]
	100	64±3 [c]	96±6 [a]	101±9 [a]	61±5 [b]	97±5 [a]	94±6 [a]	101±5 [a]	104±3 [a]
	1000	32±2 [d]	31±3 [b]	107±6 [a]	35±2 [c]	99±8 [a]	79±6 [b]	110±7 [a]	87±5 [b]
Caco-2	C	100±9 [a]	100±9 [a]	100±8 [a]	100±10 [a]	100±7 [a]	100±8 [a]	100±8 [a]	100±10 [a]
	10	100±6 [a]	101±6 [a]	98±7 [a]	95±8 [a]	109±7 [b]	109±10 [a]	72±5 [b]	94±8 [a]
	100	98±7 [a]	97±11 [a]	89±4 [b]	99±9 [a]	100±7 [a]	107±8 [a]	68±4 [c]	81±7 [b]
	1000	43±3 [b]	35±3 [b]	79±5 [c]	99±6 [a]	66±4 [c]	73±6 [b]	69±5 [c]	56±5 [c]
A549	C	100±7 [a]	100±9 [a]	100±3 [a]	100±7 [a]	100±7 [a]	100±8 [a]	100±3 [a]	100±7 [a]
	10	99±7 [a]	106±5 [a]	92±7 [a]	109±8 [a]	94±4 [a]	102±10 [a]	82±5 [b]	101±7 [a]
	100	94±5 [a]	99±5 [a]	95±6 [a]	97±7 [a]	94±5 [a]	107±6 [a]	90±5 [c]	99±6 [a]
	1000	65±3 [b]	63±3 [b]	89±5 [b]	74±5 [b]	76±3 [b]	67±4 [b]	82±7 [b]	77±5 [b]
OE-33	C	100±6 [a]	100±3 [a]	100±10 [a]	100±10 [a]	100±6 [a]	100±3 [a]	100±10 [a]	100±10 [a]
	10	98±8 [a]	96±4 [a]	109±7 [a]	92±10 [a]	89±3 [b]	97±3 [a]	100±7 [a]	86±8 [b]
	100	86±7 [b]	83±2 [b]	105±4 [a]	90±5 [a]	101±8 [a]	97±4 [a]	99±6 [a]	81±8 [b]
	1000	41±3 [c]	5±1 [c]	85±5 [b]	15±2 [b]	76±6 [c]	66±2 [b]	94±7 [a]	66±3 [c]
T24	C	100±6 [a]	100±5 [a]	100±11 [a]	100±8 [a]	100±6 [a]	100±5 [a]	100±11 [a]	100±8 [a]
	10	97±8 [a]	103±4 [a]	95±17 [a]	77±7 [b]	94±8 [a]	104±6 [a]	100±10 [a]	72±7 [b]
	100	87±7 [b]	102±4 [a]	99±16 [a]	52±5 [c]	100±8 [a]	103±7 [a]	108±9 [a]	75±5 [b]
	1000	20±2 [c]	2±0 [b]	56±5 [b]	0±3 [d]	93±8 [a]	71±5 [b]	113±10 [a]	49±5 [c]

Values are expressed as the mean ± SD. Different letters within a column and cell line indicate significant differences ($p<0.05$).

Concerning CA and FA, both compounds were less effective than the previous caffeoylquinic esters, since in general only the highest dose tested reduced cell viability (Table 17). Only OE-33 cells were slightly more susceptible, with 100 μM CA also decreasing cell viability and, interestingly, 10 μM FA at short incubation time. Normal, non-cancer cells were also affected by CA, showing a reduced viability after 2 h incubation with 10 μM CA. However, low doses of CA had no antiproliferative effects in any of the studied cell lines, except in T24 cells after treatment for 24 h. On the contrary, FA showed decreased proliferation of all the cancer cell lines, with the 10 μM concentration having a significant antiproliferative effect in Caco-2 and A549 cells after 2 h, and in OE-33 and T24 at longer incubation time (24 h), pointing to an increased antiproliferative effect of the methylated molecule as compared to unmethylated CA.

The microbial metabolites DHCA and DHFA also showed a limited activity reducing cell viability since, in general, only the highest concentrations decreased the viability of all cell lines (Table 18). Exceptionally, OE-33 cells showed an apparent increased viability with 10-100 μM DHCA after 24 h incubation, the 100 μM dose also increasing cell proliferation. Low concentrations of DHCA (10 μM) decreased proliferation of Caco-2 and A549 cells, also affecting normal CCD-18Co cells. Contrary to FA, the reduced methylated molecule DHFA only had a significant antiproliferative effect on urinary bladder T24 cells (Table 18).

Both coffee and yerba mate contain caffeine in relevant amounts (7-9 mg/g in mate and 11.5-13 mg/g coffee; Baeza et al., submitted), which is present in the tested extracts. Therefore, the potential antiproliferative effect of this methylxanthine was also tested. Only the highest concentration showed some effect reducing cell viability in all but Caco-2 cells, also having a modest antiproliferative effect in A549 and T24 cells after incubation for 24 h (Table 19). However, the 10 μM dose, a concentration found in plasma after consumption of a single cup of coffee (Martinez-Lopez et al., 2014), had no effect on cell viability nor proliferation.

Table 18. Effect of dihydrocaffeic and dihydroferulic acids on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33 and T24) human cell lines.

Cell lines	Treatment (μM)	Dihydrocaffeic acid				Dihydroferulic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100±5 ^{a}	100±7 ^{a}	100±10 ^{a}	100±10 ^{a}	100±8 ^{a}	100±7 ^{a}	100±8 ^{a}	100±8 ^{a}
	10	87±5 ^{b}	97±7 ^{a}	79±7 ^{b}	94±10 ^{ab}	99±6 ^{a}	102±8 ^{a}	94±10 ^{a}	104±9 ^{a}
	100	75±3 ^{c}	106±5 ^{a}	74±8 ^{b}	85±9 ^{b}	99±6 ^{a}	100±4 ^{a}	98±10 ^{a}	101±10 ^{a}
	1000	79±5 ^{c}	13±1 ^{b}	65±5 ^{c}	28±3 ^{c}	89±6 ^{b}	72±5 ^{b}	102±9 ^{a}	100±6 ^{a}
Caco-2	C	100±10 ^{a}	100±10 ^{a}	100±8 ^{a}	100±8 ^{a}	100±10 ^{a}	100±4 ^{a}	100±8 ^{a}	100±8 ^{a}
	10	92±7 ^{a}	102±8 ^{a}	75±5 ^{b}	106±8 ^{a}	91±7 ^{ab}	93±8 ^{a}	101±10 ^{a}	106±8 ^{a}
	100	91±5 ^{a}	99±9 ^{a}	77±8 ^{b}	98±9 ^{a}	90±5 ^{b}	83±8 ^{b}	107±9 ^{a}	98±9 ^{a}
	1000	75±3 ^{b}	97±10 ^{a}	69±5 ^{b}	98±9 ^{a}	75±3 ^{c}	73±5 ^{c}	99±7 ^{a}	98±9 ^{a}
A549	C	100±7 ^{a}	100±8 ^{a}	100±3 ^{a}	100±3 ^{a}	100±2 ^{a}	100±7 ^{a}	100±9 ^{ab}	100±8 ^{a}
	10	97±6 ^{a}	96±7 ^{a}	86±7 ^{b}	82±5 ^{b}	100±4 ^{a}	96±9 ^{a}	106±12 ^{ab}	100±10 ^{a}
	100	102±9 ^{a}	100±6 ^{a}	92±6 ^{ab}	90±5 ^{a}	98±5 ^{a}	92±7 ^{a}	93±9 ^{a}	99±6 ^{a}
	1000	78±3 ^{b}	80±5 ^{b}	86±8 ^{b}	82±7 ^{b}	79±5 ^{b}	79±4 ^{b}	113±11 ^{b}	76±8 ^{b}
OE-33	C	100±6 ^{a}	100±3 ^{a}	100±4 ^{a}	100±10 ^{a}	100±3 ^{a}	100±3 ^{a}	100±6 ^{a}	100±10 ^{a}
	10	89±12 ^{a}	107±4 ^{b}	93±8 ^{a}	98±9 ^{a}	101±4 ^{a}	96±5 ^{ab}	103±4 ^{a}	96±10 ^{a}
	100	91±8 ^{a}	125±4 ^{c}	83±8 ^{a}	117±12 ^{b}	99±4 ^{a}	92±5 ^{b}	98±8 ^{a}	104±8 ^{a}
	1000	100±8 ^{a}	10±0 ^{d}	73±5 ^{b}	30±4 ^{c}	86±4 ^{b}	61±4 ^{c}	88±5 ^{b}	76±6 ^{b}
T24	C	100±6 ^{a}	100±5 ^{a}	100±5 ^{a}	100±10 ^{a}	100±7 ^{a}	100±5 ^{a}	100±11 ^{a}	100±8 ^{a}
	10	96±6 ^{a}	105±5 ^{ab}	96±9 ^{a}	108±6 ^{a}	92±8 ^{ab}	100±7 ^{a}	110±11 ^{a}	80±6 ^{b}
	100	80±6 ^{b}	110±3 ^{b}	122±7 ^{b}	116±10 ^{a}	91±7 ^{ab}	93±8 ^{a}	97±13 ^{a}	79±5 ^{b}
	1000	15±1 ^{c}	7±1 ^{c}	13±1 ^{c}	2±1 ^{b}	86±8 ^{b}	74±7 ^{b}	91±8 ^{a}	52±5 ^{c}

Values are expressed as the mean ± SD. Different letters within a column and cell line indicate significant differences ($p<0.05$).

Table 19. Effect of caffeine and chlorogenic acid at the indicated concentrations, for 2 and 24 h, on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33 and T24) human cell lines.

Cell lines	Treatment (μM)	Caffeine			
		Viability		Proliferation	
		2 h	24 h	2 h	24 h
CCD-18Co	C	100±6 ^{a}	100±7 ^{a}	100±10 ^{a}	100±10 ^{a}
	10	101±6 ^{a}	99±8 ^{a}	106±6 ^{a}	98±8 ^{a}
	100	100±8 ^{a}	98±7 ^{a}	103±8 ^{a}	106±8 ^{a}
	1000	94±6 ^{a}	86±6 ^{b}	110±10 ^{a}	107±9 ^{a}
Caco-2	C	100±5 ^{a}	100±7 ^{a}	100±8 ^{a}	100±8 ^{a}
	10	101±8 ^{a}	110±8 ^{a}	101±8 ^{a}	99±8 ^{a}
	100	98±4 ^{ab}	101±5 ^{a}	116±5 ^{b}	108±11 ^{a}
	1000	91±6 ^{b}	109±6 ^{a}	98±8 ^{a}	106±9 ^{a}
A549	C	100±6 ^{a}	100±7 ^{a}	100±8 ^{a}	100±8 ^{a}
	10	104±9 ^{a}	94±4 ^{ab}	84±6 ^{b}	117± ^{b}
	100	110±9 ^{a}	90±5 ^{b}	79±6 ^{bc}	106±7 ^{a}
	1000	109±5 ^{a}	91±3 ^{b}	73±4 ^{c}	82±7 ^{b}
OE-33	C	100±6 ^{a}	100±8 ^{a}	100±7 ^{ab}	100±10 ^{a}
	10	100±5 ^{a}	91±5 ^{ab}	116±5 ^{a}	103±10 ^{a}
	100	108±6 ^{a}	95±7 ^{a}	98±10 ^{b}	90±7 ^{a}
	1000	99±5 ^{a}	82±5 ^{b}	99±11 ^{ab}	92±10 ^{a}
T24	C	100±4 ^{a}	100±5 ^{a}	100±5 ^{a}	100±10 ^{a}
	10	96±3 ^{a}	103±4 ^{a}	120±10 ^{b}	115±8 ^{a}
	100	101±7 ^{a}	104±3 ^{a}	101±13 ^{a}	111±9 ^{a}
	1000	99±6 ^{a}	84±6 ^{b}	114±7 ^{ab}	67±6 ^{b}

Values are expressed as the mean ± SD. Different letters within a column and cell line indicate significant differences ($p<0.05$).

As it can be seen, all the four cancer cell lines showed different response to treatments with GCBE, YME and the major phenolic constituents and metabolites. Looking only at their response to the physiologically plausible concentration of 10 μg/mL or 10 μM, in terms of cell viability OE-33 were more susceptible, with GCBE, YME, 5-CQA, FA and DHCA decreasing the viability of this oesophageal cancer cell line. However, only FA showed antiproliferative effect in these cells. The other cell lines were less susceptible, with only YME and, at the most GCBE (in T24 cells), reducing cell viability (Table 20).

Table 20. Effect of 10 µg/mL GCBE and YME or 10 µM 5-CQA, 3,5-DCQA, CA, FA, DHCA, DHFA, and caffeine decreasing viability and proliferation of the studied cell lines after short (S, 2 h) or long (L, 24 h) incubation times. (-, no effect).

	Caco-2		A549		OE-33		T24		CCD-18Co	
	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation
GCBE	-	S	-	-	L	-	L	-	-	-
YME	S,L	S	S,L	L	S,L	-	S,L	L	S,L	-
5-CQA	-	-	-	-	S,L	-	-	-	S	-
3,5-DCQA	-	L	-	-	-	-	-	L	-	-
CA	-	-	-	-	-	-	-	L	S	-
FA	-	S	-	S	S	L	-	L	-	-
DHCA	-	S	-	S,L	-	-	-	-	S	S
DHFA	-	-	-	-	-	-	-	L	-	-
CAF	-	-	-	-	-	-	-	-	-	-

When considering cell proliferation, however, A549 was affected by YME, FA and DHCA, but interestingly proliferation of Caco-2 and T24 cells could be significantly decreased by up to five out of the eight extracts/compounds tested. Thus, Caco-2 cells were susceptible to 10 µg/mL GCBE and YME, and 10 µM 3,5-DCQA, FA and DHCA, while T24 cells were affected by 10 µg/mL YME, and 10 µM 3,5-DCQA, CA, FA and DHFA (Table 20).

A similar analysis of the results show that YME was the most active compound, the 10 µg/mL dose reducing viability of all the tested cancer cell lines and decreasing proliferation of all but OE-33 cell lines. Among the pure polyphenols, FA seemed the most active, with antiproliferative effects in the four cancer cell lines. However, while the monocaffeoylquinic acid 5-CQA had no antiproliferative action, 3,5-DCQA showed antiproliferative effect in Caco-2 and T24 cells (Table 20).

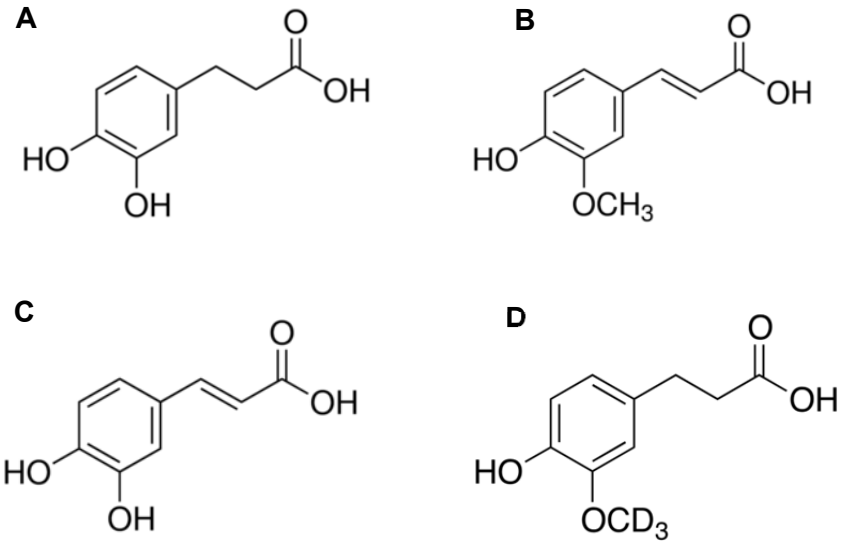


Figure 37. Chemical structures of caffeic acid (A), ferulic acid (B) and the reduced microbial metabolites dihydrocaffeic (C) and dihydroferulic (D) acids.

It is interesting noting the different activity of CA, FA and their reduced microbial metabolites DHCA and DHFA (Figure 37). As just mentioned, 10 µM FA reduced proliferation of all cancer cells, also decreasing OE-33 cell viability; in contrast, the methoxylated molecule, CA, only decreased proliferation of T24

cells. On the contrary, reduction of the molecule by the microflora led to a DHFA molecule that only affected proliferation of T24 cells, whilst DHCA showed antiproliferative effect in Caco-2 and A549 cells, also decreasing viability of OE-33 cells. Finally, caffeine had no effect on cell viability and proliferation.

However, tested compounds also affected the non-cancer cell line CCD-18Co. Only DHCA reduced the proliferation of these fibroblast colonic cells, although YME, 5-CQA, CA, and DHCA decreased cell viability at the 10 µg/mL or 10 µM dose (Table 20). Therefore, we studied the potential cytotoxicity of these compounds at lower concentrations in CCD-18Co. Also, since 10 µg/mL YME was very effective decreasing cell viability and proliferation the cytotoxic effect of lower concentrations of this extract was also studied.

Cytotoxicity and antioxidant effect in CCD-18Co cells

In order to further understand the potential negative effects observed in CCD-18Co cells with the lowest dose of the tested compounds, cytotoxicity of YME (0.1, 1, and 10 µg/mL) and 5-CQA, CA and DHCA (0.1, 1 and 10 µM) after 2 and 24 h treatment was evaluated by the LDH method. The percentage of LDH indicates the cytotoxicity of the test compounds, so that the higher the values with respect to the control, the higher the cytotoxicity. None of the compounds induced cytotoxicity in CCD-18Co cells. On the contrary, a cytoprotective effect was observed when cells were incubated with 1 and 10 µg/mL YME, with significant reduction of LDH leakage from damaged cells (about 20 and 10% at 1 µg/mL and 10 and 35% at 10 µg/mL, after 2 and 24 h incubation, respectively) (Figure 38). Accordingly, treatment with 10 µM 5-CQA induced a reduction in % LDH of 15 and 7%, at 2 and 24 h, respectively, with a similar reduction after 2 h with 1 µM 5-CQA. CA at the three concentrations studied induced a reduction in %LDH after 2 h treatment but not at longer incubation time (24 h). When CCD-18Co cells were treated with DHCA no changes in cytotoxicity were observed, except for a decrease in LDH % with the treatment at 0.1 µM for 2 h.

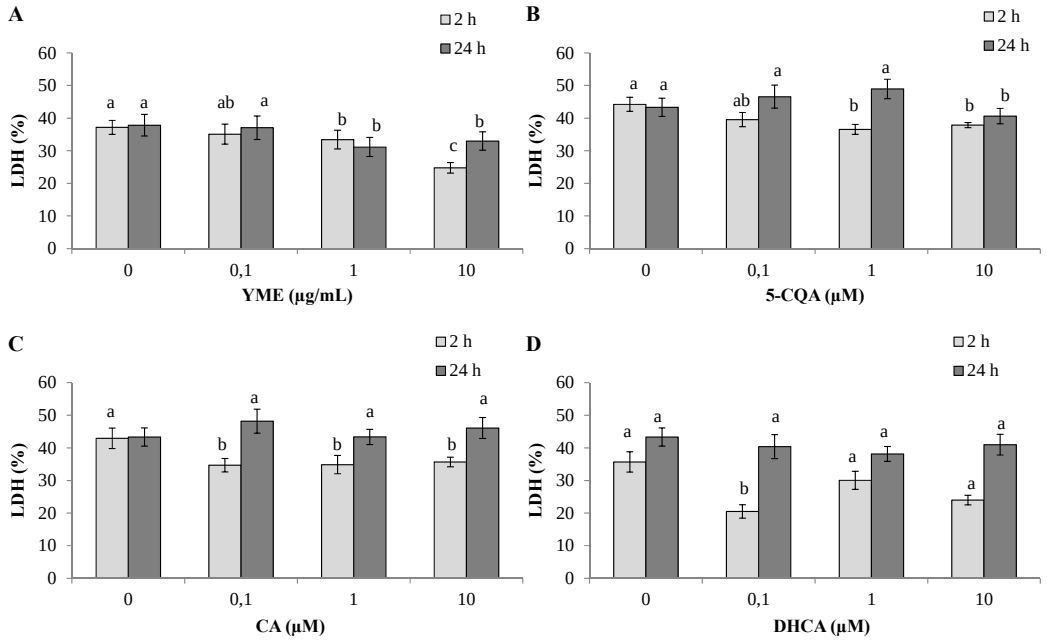


Figure 38. Cytotoxicity studies in human healthy colonic CCD-18Co cells after treatment with YME (A), 5-CQA (B), CA (C) and DHCA (D) for 2 and 24 h. Different letters within the same incubation time denote statistically significant differences ($p<0.05$). Results are the mean ± SD (n=4).

Treatment of CCD-18Co cells with YME resulted in a decrease in the generation of reactive oxygen species (ROS) with the 1 and 10 µg/mL doses, while 0.1 µg/mL had no effect (Figure 39a). Similarly, 0.1 µM 5-CQA, CA, and DHCA had no effect on ROS levels, while the higher concentrations were able to significantly decrease ROS generation from 39-72 % (with respect to basal conditions) after 2 h.

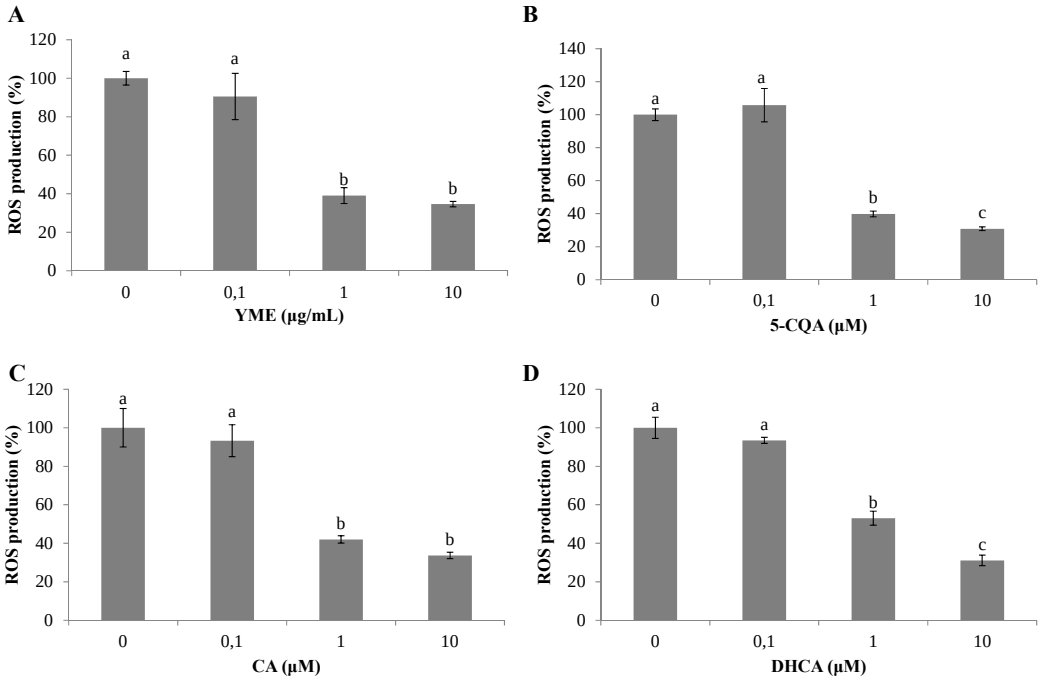


Figure 39. ROS generation in CCD-18Co cells after 2 h treatment with YME (A), 5-CQA (B), CA (C), and DHCA (D). Different letters denote statistically significant differences ($p<0.05$). Results are the mean ± SD (n=4).

Effect of YME on cell cytotoxicity and ROS production

Cytotoxicity experiments using the LDH assay were also carried out with physiological doses (0.1-10 µg/mL) of YME in the four cancer cell lines (Figure 40). After 2 h treatment, YME only induced a slight yet statistically significant cytotoxic effect in OE-33 cells at 1 and 10 µg/mL of YME (Figure 40C). No cytotoxicity was observed in the rest of the other studied cancer cell lines, with a cytoprotective action of 1 and 10 µg/mL YME in colonic Caco-2 cells, and 0.1 and 10 µg/mL in T24 bladder cells.

The generation of ROS after 2 and 24 h treatment with YME is shown in Figure 41. YME significantly decreased ROS generation in a dose-dependent manner in all cell lines, the 0.1 µg/mL dose being able to decrease ROS production in Caco-2 and T-24 cells as compared to the respective untreated controls (Figure 41A and D).

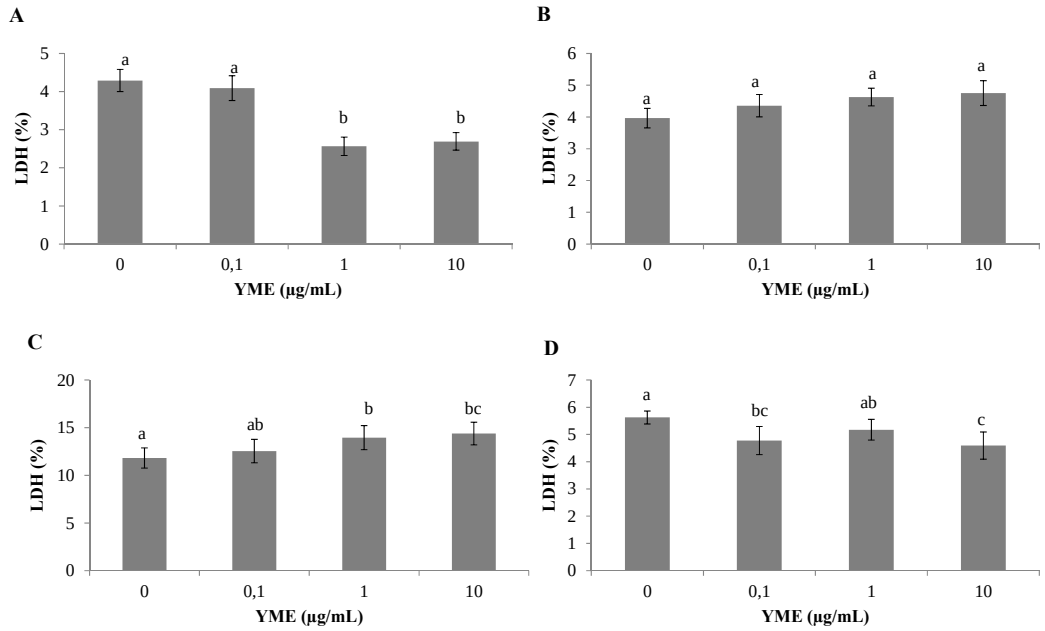


Figure 40. Cytotoxicity studies in tumour cell lines treated with YME for 2 h. (A) Caco-2 cells; (B) A549 cells; (C) OE 33 cells; (D) T-24 cells. Different letters denote statistically significant differences ($p<0.05$). Results are the mean ± SD (n=4).

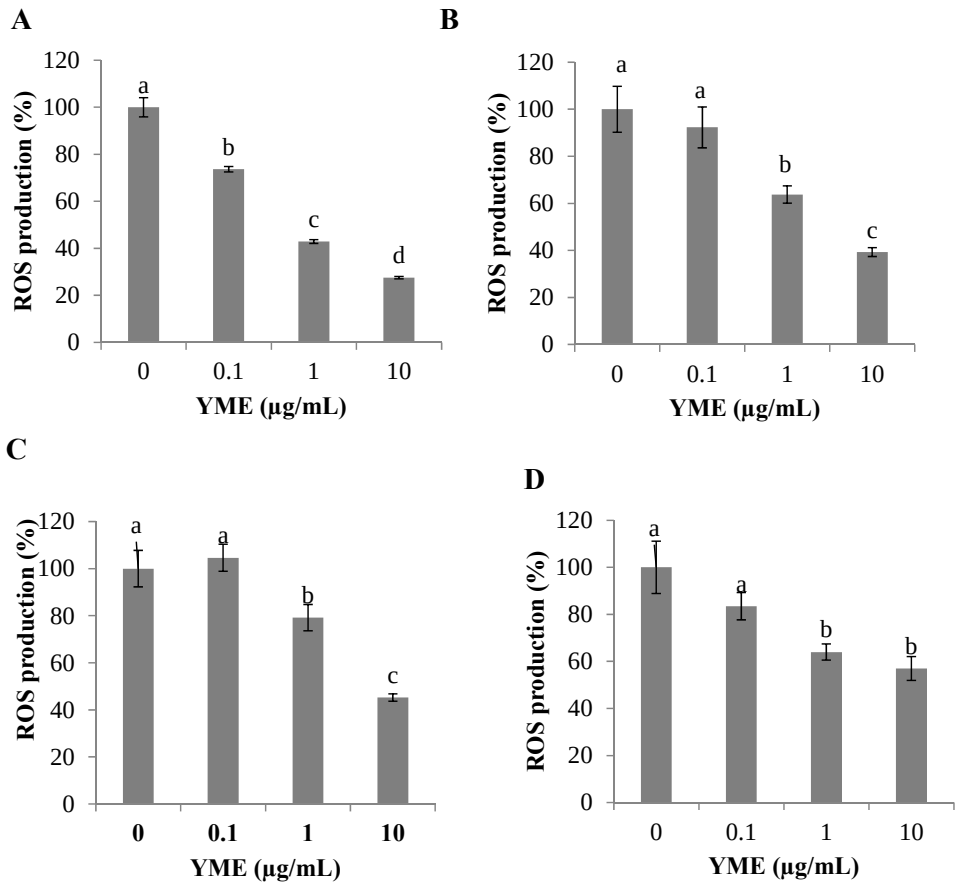


Figure 41. ROS production in Caco-2 (A), A549 (B), OE-33 (C), and T-24 (D) cells treated with YME for 2 h. Different letters denote statistically significant differences ($p<0.05$). Results are the mean ± SD (n=4).

Discussion

Cell viability, often defined as the number of healthy cells in a sample, plays a fundamental role in all forms of cell culture, and is frequently the main purpose of toxicity assays. Alternatively, cell viability can be used to correlate cell behavior to cell number providing an accurate picture of cell activity. In contrast, cell proliferation considers the increase in the number of cells as a result of cell growth and division. Cancer is associated with oxidative stress and uncontrolled cell proliferation (Nkondjock, 2009). Dietary polyphenols are antioxidants and many of them have shown to exert potential anticarcinogenic activities (Surh, 2003; Ramos, 2008; Araujo et al., 2011). Thus, flavonols like quercetin present in apple, onions and many other foodstuffs, have shown to regulate proliferation and induce cell death of human hepatoma cells (Granado-Serrano et al., 2008; 2010a). Tea flavanols (epigallocatechin, epigallocatechin gallate, etc.) suppressed the growth of different cancer cell lines (Nichenametla et al., 2006; Granado-Serrano et al., 2007; de Mejia et al., 2010; Ramos et al., 2011). Also, cocoa phenolic compounds (epicatechin, procyanidin B2) and cocoa extracts are potent inhibitors of tumor initiation *in vivo* and present antiproliferative activities against tumor cells *in vitro* (Granado-Serrano et al., 2009; Rodriguez-Ramiro et al., 2011c). Identifying dietary strategies to control the proliferation of cancer cells may contribute to a preventive therapy against the onset and progression of the illness. In this sense, highly and regularly consumed foods rich in polyphenols such as coffee in industrialized countries and yerba mate in South America and other parts of the world, are particularly interesting.

The cell lines selected for the present study are representative of some types of cancer associated to coffee or mate consumption. A meta-analysis of the effect of coffee intake and lung cancer found that high consumption may increase the risk of lung cancer [relative risk (RR) 1.27, 95% confidence interval (CI) 1.04-1.54], although a decreased risk was found in non-smokers (RR 0.78, 95% CI 0.60-1.00) (Tang et al., 2010). A case-control study in Uruguay also showed an increased risk of small cell lung cancer [odds ratio (OR) 2.9, 95% CI 1.3-6.2] with mate consumption, although pulmonary adenocarcinoma was not associated with mate drinking (De Stefani et al., 1996).

A recent case-control study in Latin America, where cancers of the upper aero-digestive tract (UADT, oral cavity, oropharynx, larynx, oesophagus) have

specially high incidence, found a significant effect of mate consumption only on oesophagus cancer (OR 3.81, 95% CI 1.75-8.30) (Szymariska et al., 2010). This was confirmed in a meta-analysis of studies on the consumption of mate in patients with oesophageal squamous cell carcinoma that found an increased risk in patients with high mate intake (OR 2.76, 95% CI 1.33-5.73) compared to low consumption (OR 1.84, 95% CI 1.12-3.00) (Andrici and Eslick, 2013). High temperature of mate consumption was associated with increased cancer risk (Sewram et al., 2003), although results in the mentioned case-control study were not conclusive (Szymariska et al., 2010). However, a synergistic effect between mate, alcohol and tobacco is clear and has been reported to represent a 7-fold increase in oesophageal cancer risk (Sewram et al., 2003; Loria et al., 2009). Also, mate was associated with increased risk of UADT cancer in Brazil (RR 2.1, 95% CI 1.6-2.7) but not with coffee and tea drinking (Pintos et al., 1994), which has been recently confirmed in a large epidemiological study in nine European countries in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Zamora-Ros et al., 2014) where even a decreased risk of oral squamous cell carcinoma among male smokers has been suggested.

Coffee and mate consumption has also been associated with increased risk of bladder cancer in observational studies. Mate (OR 2.2, 95% CI 1.2-3.9) and coffee (or 1.6, 95% CI 1.2-2.3) drinking were strongly associated with bladder cancer in the Uruguayan population (De Stefani et al., 2007). Again, high temperature and tobacco were factors increasing the risk, since mate intake showed a strong association with bladder cancer when consumed by ever-smokers with “bombilla” (OR 3.77, 95% CI 1.17-12.1) but not when consumed by never-smokers or as “mate cocido” (Bates et al., 2007). On the contrary, a recent meta-analysis showed a higher risk associated to coffee consumption in non-smokers (OR 1.72, 95% CI 1.25-2.35) compared to smokers (OR 1.24, 95% CI 0.91-1.70) (Wu et al., 2015).

Finally, several recent case-control studies and meta-analysis have shown a reduced risk of colorectal cancer with coffee drinking, with OR of 0.74-0.78 in case-control studies and RR of 0.93-0.95 in prospective cohort studies (Akter et al., 2016; Gan et al., 2016; Schmit et al., 2016). This beneficial effect of coffee intake has been extended to patients with stage III colon cancer, with a significantly reduced cancer recurrence and mortality in patients consuming 4 cups/d [hazard ration (HR) 0.58, 95% CI 0.34-0.99] (Guercio et al., 2015).

However, studies on the effect of mate intake and colon cancer are limited to a prospective study in Brazil that found that colon cancer mortality was positively associated with mate consumption (Sichieri et al., 1996), although yerba mate tea has shown to reduced colon carcinogenesis in rats (Zapaterini et al., 2010).

All the previous evidences, often contradictory, justify the interest of studying the effect of GCBE and YME in the selected cancer cell lines in this preliminary screening work. The wide range of concentrations of GCBE (10-1000 µg/mL) and the major phenolic constituents, metabolites and methylxanthines (10-1000 µM 5-CQA, 3,5-DCQA, CA, FA, DHCA, DHFA and caffeine) comprised from physiologically relevant concentrations (plasma levels of chlorogenic acids of 10-18.3 µM have been reported after coffee consumption; Monteiro et al., 2007; Farah et al., 2008) to supra-physiological high concentrations to test for potential pharmacologically relevant effects. Although most works in the literature use concentrations of pure phenolic compounds and/or polyphenol-rich extracts up to 200 µM or µg/mL, respectively (Seeram et al., 2005; Kim et al., 2006; Granado-Serrano et al., 2009; Wang et al., 2012; Venkatesan et al., 2016), it is not uncommon the use of concentrations up to 500-650 µM or µg/mL of pure compounds or extracts, respectively (de Mejia et al., 2010; Mateos et al., 2013; Caló and Marabini, 2014), or even supra-physiological levels as high as mM concentrations of pure polyphenols or 100 mg/mL of extracts (Iwai et al., 2004; Peng et al., 2013; Yang et al., 2015).

Our results show that the two extracts affected differently the viability and proliferation of the four cancer cell lines, with YME being more effective even at the lowest dose (10 µg/mL). Although GCBE and YME are rich in hydroxycinnamic acids and methylxanthines, there are quantitative differences, with coffee having more caffeine than mate (up to 13.07 mg/g vs. 8.95 mg/g in mate) and more cinnamoylquinic acids (up to 65.5 mg/g in GCBE and 57.2 mg/g in YME). However, YME is richer in dicaffeoylquinic acids (up to 16.6 mg/g vs. 5.3 mg/g in coffee) (Baeza et al., 2014; 2016). Moreover, mate also contains important amounts of flavonols (up to 4% of total polyphenols in the YME; Baeza et al., 2016), including rutin and other quercetin-glycosides. DCQA in mate have shown to inhibit human colon cancer cells CRL-2577 and HT-29 proliferation, inducing apoptosis in a time and dose-dependent manner (Puangpraphant et al., 2011b). Also, flavonols like quercetin have well-known antiproliferative effects *in vivo* and *in vitro* (Alia et al., 2006a; Granado Serrano et al., 2006; 2008; 2010a;

Araujo et al., 2011). Yerba mate also contains saponins (Baeza et al., submitted), including ursolic acid, which has been reported to induce apoptosis in HT-29 colon cancer cells (Shen et al., 2009). Therefore, differences in the response of GCBE and YME can be due to the different composition of both extracts.

This effect of YME reducing cell viability and proliferation at a physiological relevant dose (10 µg/mL) was not directly due to a pro-oxidant effect of the extract, since ROS values decreased in all cell lines incubated with low (0.1-10 µg/mL) doses of YME (Figure 41). Similarly, such effects could not be explained by a cytotoxic activity of YME, since LDH values showed no effect (A549 cells) or even a cytoprotective activity (Caco-2 and T24 cells) of the extract, which only was slightly cytotoxic in OE-33 cells (Figure 40). Further studies are needed to explore the mechanisms of action of YME decreasing cell viability and proliferation.

Previous studies with yerba mate, either as aqueous (tea-like) or organic extracts, have shown the ability of mate to inhibit oral cancer cell proliferation (de Mejia et al., 2005), having growth inhibitory and cytotoxic effects on human colon adenocarcinoma cells, with inhibitory concentrations (IC50) of 220 and 224 µg/mL in Caco-2 and HT-29 cells, respectively, although with very different GI50 (growth inhibition, 50%) values, 1 µg/mL vs. 105 µg/mL for Caco-2 and HT-29 cells respectively, showing the different susceptibility of cancer cells (de Mejia et al., 2010. Yerba mate tea has also proved to protect against oesophageal and liver carcinogenesis induced by N-diethylnitrosamine (DEN) in Wistar rats (Silva et al., 2009). All these evidences confirm the activity of YME on different types of cancer.

The observed different susceptibility of the four cancer cell lines studied would reflect their different metabolic capacity, gene expression, differentiation, tumorigenicity, etc. Even different human colon cancer cell lines have shown to respond differently to the same phytochemical as mentioned above for Caco-2 and HT-29 cells (de Mejia et al., 2010), also observed by Ramos et al. (2011) for Caco-2 and SW480 cells. Moreover, distinct capacity to uptake and accumulate polyphenols by the different cell lines would likely affect their susceptibility to the tested extracts and phytochemicals. Caco-2 cells, with characteristics of differentiated enterocytes, are known to efficiently uptake and metabolize polyphenols (Vaidyanathan and Walle, 2003; Pereira-Caro et al., 2010; Gallardo

et al., 2016), including mono- and dicaffeoylquinic acids (Gomez-Juaristi, 2015), although the capacity of A549, OE-33 and T24 cells has not been tested.

When looking at the effect of the major phenolic compounds in YME and GCBE and their main metabolites, important differences could be observed. High doses of 3,5-DCQA were more efficient than 5-CQA reducing cell proliferation (Table 16), while at low levels (10 μM) only 3,5-DCQA was able to decrease proliferation of Caco-2 and T24 cells. However, cell viability was similarly affected by the mono- and dicaffeoylquinic acids. In agreement with our results, DCQAs have shown to decrease proliferation of human colon cells (Puangpraphan et al., 2011). Similarly, Iwai et al. (2004) reported higher antiproliferative capacity of DCQAs as compared to CQAs and FQAs in several cancer cell lines, with IC50 values ranging from 0.10-0.18 mM in KB human oral carcinoma cells to 0.56-0.76 mM in human histiocytic lymphoma U937 cells for different DCQAs as compared with IC50 values of 0.56-4.65 mM for 5-FQA and 0.14 mM (KB cells) or as high as 8.18 (lung fibroblast WI38VA cancer cells) for 5-CQA. It has to be noted the high IC50 values reported by these authors, in the mM range, much higher than the concentrations tested in the present study. The antiproliferative effects of DCQAs are not limited to those present in yerba mate, since DCQAs extracted from sweet potato leaves (3,4-, 3,5- and 4,5-DCQA) dose-dependently inhibited cancer cell proliferation, inducing apoptosis mediated by caspase 3 activation and expression of c-Jun (Jurata et al., 2007).

The higher activity of DCQA as compared with CQA and FQA suggested that the number of caffeoyl groups might be a key factor regulating the growth inhibitory activity of hydroxycinnamoylquinic estes (Iwai et al., 2004), which has also been suggested to play a role in the antimutagenic capacity of CQA derivatives (Yashimoto et al., 2002).

Both 5-CQA and 3,5-DCQA were more effective than the unesterified CA and FA, since in general the highest doses of CA reduced cell viability and proliferation (with the exception of T24 cells), while FA also required high doses to reduce cell viability (Table 17). Other authors reported a reduction of 25% cell viability of T24 cells incubated 24 h with 2000 μM FA (Peng et al., 2013), similar to the 29% decreased viability of T24 cells treated for 24 h with 1000 μM FA in the present study. Contrary to CA, FA showed an effective antiproliferative action even at the lowest dose tested (10 μM), which was able to decrease between 14

and 27% the proliferation of the tested cancer cells (Table 17). This is in contrast with that reported by Iwai et al. (2004) suggesting that methoxylation of the caffeoyl group at the C3 position would result in lower antiproliferative activity against cancer cells, although that association derived from observations in 5-FQA as compared to 3-, 4- and 5-CQA. Free, non-esterified hydroxycinnamic acids might behave differently as observed in the present study. The results with the reduced microbial metabolites DHCA and DHFA, however, are in line with that suggested by Iwai et al. (2004), since the methoxylated DHFA was in general less effective than DHCA reducing cell viability and proliferation (Table 18).

Finally, the lack of effect of caffeine on any of the studied cancer cell lines, requiring 1000 μM doses to reduce proliferation in A549 and T24 cells (Table 19), agrees with results from a cluster comparative study of 130 chemicals on a panel of 39 human cancer cell lines (Naketsu et al., 2007).

Special mention deserves the effects observed in non-cancer cells, CCD-18Co. Many phytochemicals have shown to be effective reducing viability and/or proliferation of cancer cell lines not affecting normal, non-cancer cells, which is an added value to their potential as natural chemopreventive or even chemotherapeutic agents against cancer. However, the fact that both GCBE and YME decreased viability and proliferation of CCD-18Co cells, in the case of mate even at the 10 μg/mL dose with a 51% lower viability and 12% decreased proliferation after 24 h (Table 15), points to a potential undesired cytotoxic effect in normal cells. Reductions of cell viability and proliferation were also observed with most pure polyphenols and metabolites, only the methylated molecules (FA and DHFA) having lower effect decreasing cell viability after 24 h incubation with the highest dose (1000 μM).

Similar findings were reported by other authors. IC50 values ranging from 500 μM (3,5-DCQA) to 5870 μM (5-CQA) were reported for normal diploid lung fibroblast WI38 cells (Iwai et al., 2004). In normal CCD-33Co colon cells, high concentrations (500 μg/mL) of mate tea as well as green tea and epigallocatechin gallate (500 μM) reduced growth of the non-cancer cells, although 50 μg/mL concentrations had no effect on cell viability (de Mejía et al., 2010). Puang et al. (2011) reported no cytotoxic effects of 300 μM 3,5-DCQA in CCD-33Co normal cells. In contrast, in our study the 100 μM dose of 3,5-DCQA inhibited 12% and 14% cell viability of CCD-18Co cells after 2 and 24 h treatment, respectively,

while the 1000 μM concentration reduced viability to only 34% after 2 h incubation, totally blocking cell viability after 24 h (Table 16), suggesting a strong cytotoxic effect. Such effect was more remarkable for YME, 5-CQA, CA, and DHCA, since the lowest doses (10 μg/mL YME and 10 μM of pure phenolics) showed inhibition of cell viability varying between 13% (DHCA, 2 h) and 63% (YME, 2h). However, specific studies on the cytotoxicity of these samples at lower, physiological concentrations (0.1, 1, and 10 μg/mL or μM) showed that none of the tested concentrations was cytotoxic (Figure 38), even having cytoprotective effects and reducing ROS generation (Figure 39), pointing to an antioxidant effect in CCD-18Co cells.

Different mechanisms of action have been proposed to explain the reduction in cell viability and proliferation by hydroxycinnamic acids: (i) blockage of cell cycle progression by suppressing the activation of the nuclear factor-κB (NF-κB); (ii) cell cycle arrest and induction of apoptosis; (iii) activation/deactivation of protein kinases (Bohn et al., 2014). Previous studies have reported an inhibition of NF-κB in macrophages treated with di-CQA purified from YME, as well as the induction of apoptosis by activation of caspases-8 and -3 in human colon cancer cells (HT-29 and CRL-2577) in a time and concentration dependent manner (Puangpraphant et al., 2011b). Also cycle arrest in G2/M phase and accumulation of cells in subG0 phase of cycle cells have been observed in human cervical adenocarcinoma HeLa cells incubated with coffee extract (Krstic et al., 2014), although not in human hepatoma HepG2 cells treated with 5-CQA (Ramos et al., 2011). DCQAs have shown anti-inflammatory activity by suppressing the COX-2/PGE2 and iNOS/NO pathways in macrophages and colon cancer cells (Puangpraphant et al., 2011b). Topoisomerase II, a nuclear enzyme that regulates DNA metabolism and one of the targets in the development of drugs against cancer, is inhibited *in vitro* by yerba mate (de Mejia et al., 2005).

Data on ROS production when cancer cells were incubated with YME and when normal cells were incubated both YME or its CQA derivatives confirmed our previous results with 5-CQA, 3,5 DCQA (Baeza et al., 2014), DHCA and DHFA (Baeza et al., 2016) or other herbal extracts which contain these phenolic compounds such as green coffee (Baeza et al., 2014) and *Corema album* extracts (León-González et al., 2012). The antioxidant activity might therefore

contribute to reduce the oxidative stress associated with cancer initiation and progression.

Conclusions

In conclusion, consumption of green coffee and especially yerba mate, which are rich in bioactive compounds such as CQA and DCQA derivatives, do not increase proliferation of cancer cells, negatively affecting cell viability and proliferation in a dose- and time-dependent manner. 5-CQA and 3,5-DQA were the most potent anti-proliferative phenols of all the studied compounds, their synergistic effect, together with the potential contribution of other bioactive compounds likely explaining the stronger activity of YME. At physiological doses YME do not have a cytotoxic effect in cancer and normal, non-cancer cells, reducing basal ROS production showing antioxidant capacity. Therefore, yerba mate might have interesting anti-proliferative potential in cancer prevention, deserving further studies to confirm this relationship as well as the subjacent mechanisms of action.

General Discussion

6. General Discussion

Every single individual considers health as one of the major goals to attain in life. Health, as defined by the World Health Organization in 1948 (a definition never revised since its adoption by WHO) is “a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity” (WHO, 1948). Food is one of the major providers of health, not only because foods provide the basic nutrients to support life, food also affords pleasure and well-being when consumed alone or socially. Moreover, beyond covering basic nutritional requirements to avoid diseases associated with nutritional deficits, foods in a proper diet, together with a healthy lifestyle, can also promote health and reduce the risk of disease. Amounting scientific evidences support the physiological (and psychological) beneficial effects of some foods and food components, which is the basis of the concept of “**Functional Foods**” (and its extension to “Functional Food Ingredients”). This concept was initially proposed in the 1980s in Japan, considering that foods specifically developed to promote health and reduce the risk of disease would improve quality of live and increase life expectancy.

Although there is no legal definition for Functional Foods by the European Union or the U.S. Food and Drug Administration (FDA), the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) proposed a working definition of functional food as “a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement” (European Commission, 2010). Thus, functional foods would contain not only nutrients (vitamins, minerals, protein, fat, carbohydrates), but also biologically active substances such as phytochemicals (polyphenols, methylxanthines, carotenoids, phytoestrogens, melanoidins, capsaicins, etc.), bioactive peptides, mono- and polyunsaturated fatty acids, prebiotics (including dietary fibre), probiotics, and a long *et cetera*.

The hypothesis that foods and diet can promote health has evolved in the evolution of nutritional sciences from the classical concept of basic *nutritional adequacy* to avoid nutrient deficiencies to more advanced concepts like *positive* nutrition, *optimal* nutrition and more recently *personalized* nutrition, the latter

considering that the specific dietary requirements of individuals or groups of individuals would be dictated by factors like genetic background, epigenetics modifications, chronobiology, microbiota composition, or lifestyle habits, among others.

There is an increasing awareness by consumers of the importance of an adequate diet and the potential health promoting effects of certain foods and food components like fruits, vegetables, wholegrain cereals or olive oil and the phytochemicals and bioactive compounds they contain. This has led to an increased demand for health-promoting functional foods and **nutraceuticals** (these being foods or part of foods with proven safety and health effects to be administered orally in a non-food matrix or in non-conventional food formats and in amounts exceeding those achievable from normal foods with the frequency required to promote health; Palthur et al., 2010). This increased demand, in turn, has boosted the research on bioactive food components and the development of a profitable market with a myriad of foods, food ingredients and nutraceuticals offered to consumers in industrialized countries.

To cover the increasing demand for functional food ingredients and nutraceuticals, new sources of bioactive food components are constantly searched. Among such promising sources, many are plant-derived products including alternative crops such as Andean pseudocereals like chia, quinoa, amaranth, buckwheat, etc., underutilized legumes like black gram, green gram, horse gram, moth bean, and many others, halophytic plants or algae, including microalgae, although animal based products are also gaining interest. However, agroindustry by-products are perhaps the most promising source of bioactive components. Most food by-products would contain important amounts of residual components with biological activity, like dietary fibre, oligosaccharides, polyphenols, carotenoids, terpenoids, fatty acids, proteins and peptides, etc. Exploitation of such by-products adds value to materials that re-enter the food chain to provide ingredients or nutraceuticals with enhanced economic value, instead of being used as feed for livestock or in aquaculture (the traditional, low-value utilization of most by-products from the food industry), as compost, or simply being discarded with the consequent environmental impact. Utilization of agro-industrial by-products and underutilized crops to reduce food wastage and environmental contamination, extending their utilization in the food chain (or in other industries like cosmetics, pharmaceuticals, food packaging, etc.), and

adding value to these materials are key priorities of most **Bioeconomy** strategies in industrialized countries, including the *Spanish Bioeconomy Strategy* recently launched by the Ministry of Agriculture, Food and Environment (MAGRAMA), and the Ministry of Economy and Competitivity (MINECO).

In the present Thesis, one of the major food by-products in Spain and in other European countries (like France, Italy or Greece), the United States, South Africa, or Latin American countries like Chile or Argentina has been addressed. Grape is an important crop in Europe, with close to 30 million tons produced in 2013 (FAOSTAT-FAO Statistical Database, 2016). These figures support the selection of this by-product, considering that approximately 80% of grapes are used in wine-making and that 20-25% remains as winery wastes. But also, wine by-products are an excellent source of bioactive components, including dietary fibre and polyphenols (Bravo and Saura-Calixto, 1998; Teixeira et al., 2014). Grape by-products have shown to have important beneficial effects in different chronic pathologies associated with oxidative stress, especially in cardiovascular disease, decreasing the levels of total and oxidized LDL, inhibiting platelet aggregation or improving endothelial function (Dohadwala and Vita, 2009; Karthikeyan et al., 2009; Xia et al., 2010), as well as in inflammation and obesity (Décordé et al., 2009; Hogan et al., 2010; Wightman and Heuberger, 2015), among others.

Two other products have also been studied, green coffee and yerba mate. Coffee and mate, together with tea, are the most consumed drinks in the world after water. In consequence, their potential effects on health, either beneficial or detrimental, are of upmost interest at the public health level. The implications of (roasted) coffee on health have been extensively studied, and mate has also gained attention in recent years. Green coffee, however, has been less studied, although its consumption is steadily increasing alone as an infusion or blended with roasted coffee beans as a healthier alternative to traditional (roasted) coffee. Both beverages, green coffee and yerba mate, besides sharing a similar phenolic composition, have important health effects associated to their high content in hydroxycinnamic acids (chlorogenic acids). In the last years, our research team has been studying the effect of these two beverages, having shown important health beneficial effects as evidenced in human intervention trials in healthy and at risk volunteers (hypercholesterolemic subjects). Realistic consumption of these two beverages showed remarkable hypotensive, hypolipemic, hypoglycaemic,

anti-oxidant, anti-inflammatory, and anti-obesity effects (Martinez-Lopez, 2014; Sarria et al., in press; Martinez-Lopez et al., unpublished results; Sarria et al., submitted). Similarly, mechanistic studies in cell models showed the ability of green coffee beans and yerba mate extracts and their major phenolic constituents and metabolites protecting human hepatic HepG2 cells from oxidative damage (Baeza et al., 2014, 2016).

Although green coffee and traditionally yerba mate are consumed as beverages, their use as ingredients in nutraceuticals has increased in recent years, mostly associated to their potential anti-obesity effects (Vinson et al., 2012). Grape skins, grape seeds or grape pomace have also been used as functional ingredients and many nutraceuticals contain these winery by-products as well as pure phenolic compounds extracted from grapes (deserving special mention resveratrol), these by-products having also been used in the pharmaceutical and cosmetic industries.

There are many studies in the scientific literature on the composition, use and properties of grape by-products; however, the present work provides new data on the phenolic composition and biological properties of a red grape pomace obtained from a Spanish winery from Ribera del Duero. This pomace had the peculiarity of lacking in its composition monomeric flavanols (catechin, epicatechin), which are commonly present in this kind of products since they are abundant in grape seeds (Ribeiro et al., 2015). Another striking finding was the low content in anthocyanins, also usually in relatively high amounts in red grape by-products since these flavonoids are present in the skin of red grape varieties (Kammerer et al., 2004). Although we cannot totally rule out partial extraction and/or quantification of flavanols and anthocyanins under the chromatographic and mass spectrometric conditions used for their quantitative and qualitative analysis, other factors might have also determined the limited levels found the red grape pomace. It is well-known that the composition of phenolic extracts from winery wastes depends on the grape cultivar, agronomic and climatic conditions, degree of ripeness at recollection, the tissue considered (seeds, skins, stems, leaves, etc.), or the conditions during industrial processing for wine-making, but also those applied to the by-products. Thus, conditions during drying, milling or storage of the waste products would affect the final phenolic composition since polyphenols, especially anthocyanins, are highly reactive species, easily degraded. But also further extraction of other co-products for different uses (like

grape seed oil or other polyphenols such as catechins or anthocyanins themselves, the latter used as colorants, resveratrol, etc.) might account for the reduced amounts of flavanols and anthocyanins found in the present work, resulting in a product with a high content in phenolic acids (hydroxybenzoic acids) and flavonols as reported in **Chapter 1**. Therefore, of the total phenolic content in RGP (2 mg/g dry matter, Table 9) 83.3% corresponded to hydroxybenzoic acids, 15.4% were flavonols and only 1.3% were anthocyanins.

Although collateral to the general interest of this thesis, devoted to the study of the biological activity of phenolic-rich products, it was necessary to ascertain the stability of this specific red grape pomace under controlled storage conditions considering its potential future use as a functional food ingredient or nutraceutical. Establishment of a recommended maximum storage time of 6 months would help to ensure the product will preserve its biological properties in optimal conditions when consumed, being microbiologically safe for even longer times (9 months). The fact that temperature (4 or 25 °C) did not affect the phenolic composition and antioxidant capacity of the grape pomace is also of interest for the industry, since cold storage is more expensive and requires specific infrastructures, in contrast to handling and stowage of the product at room temperature.

The biological activity of phenolic compounds greatly depends on their bioavailability. A great proportion of ingested polyphenols are not absorbed in the gastrointestinal tract, reaching the large intestine where they may be metabolized by the colonic microbiota, the remaining non-absorbed and un-metabolized polyphenols being excreted in faeces. Likewise phenolic compounds absorbed in the upper gastrointestinal tract, microbial metabolites may be absorbed and further metabolized by phase II conjugating enzymes into methylated, glucuronidated and/or sulphated derivatives, reaching circulation at longer times after ingestion compared to “intestinal” metabolites (those absorbed in the stomach or small intestine). Determination of the bioavailability of phenolic compounds requires performing costly and complex acute human intervention studies, analysing metabolites in blood and/or urine samples taken at different time intervals after consumption of the polyphenol-rich foodstuff by high-throughput analytical techniques (i.e. metabolomics) to identify numerous potential metabolites and quantify them in concentrations often under the nM range.

A preliminary approximation to study the bioavailability of phytochemicals is the study of their **bioaccessibility**, i.e. the fraction available for absorption in the small intestine after digestion. *In vitro* simulated digestion mimics the conditions (pH, temperature, enzymes, presence of bile salts) in the gastric and intestinal digestion steps, and it is an accepted approach to the study of the bioaccesibility of food components.

As mentioned above, polyphenols are highly reactive compounds and digestion has shown to affect the stability of grape polyphenols, as reported in **Chapter 1**. Acid gastric conditions did not modify the composition and antioxidant activity of the red grape pomace phenolic compounds; however, the slightly alkaline intestinal pH did significantly reduce the total phenolic content of the red grape pomace and in consequence its antioxidant capacity. Enzymatic hydrolysis by pancreatin is ruled out as the cause of the observed reduction in the phenolic content, not only because the chemical structure of polyphenols clearly show they cannot be substrates of this hydrolytic enzyme, but also because changes in the phenolic composition where comparable to those in the enzyme and bile salts blank, also discarding potential interaction between bile salts and RGP polyphenols. In consequence, it was not surprising that anthocyanins were the polyphenols more extensively affected, totally disappearing after the intestinal digestion step (Table 9), since these flavonoids require acid pH to maintain the stability of their chemical structure (the flavylium cation form) (Garrido and Borges, 2013). Our results agree with those reported by other authors showing the lack of effect of gastric conditions on phenolic stability in contrast to the reduction during intestinal digestion of the content of polyphenols in different foods like pomegranate juice (Pérez-Vicente et al., 2002), green and black tea (Record and Lane, 2001), olive oil polyphenols (Pereira-Caro et al., 2012), or green coffee and yerba mate, as seen in recent studies from our research group (Baeza et al., submitted-d).

When determining the biological activity of grape pomace, assessing the effect of the complete polyphenolic extract was crucial, but also determining the contribution of its major phenolic compounds was of interest. Flavonols have been extensively studied, also by our research team (Alia et al., 2006b; Granado-Serrano et al., 2008, 2010a; Martin et al., 2010a). Besides, their lower presence in the RGP (15.4% of total phenolics) and their relative loss after *in vitro* gastrointestinal digestion (about 34% of total flavonols), drove us to focus on

phenolic acids. Of these, gallic and ellagic acids were the most abundant in RGP, accounting for 32 and 20% of the total phenolic acid content Table 9. The metabolism of ellagic acid as other ellagitannins and gallotannins has been extensively studied, and it is already well-known that these compounds are metabolized by the colonic microflora into different urolithins (Tomás-Barberán et al., 2014), being these the active circulating molecules. Therefore, we selected gallic acid (3,4,5-trihydroxybenzoic acid) as the major phenolic compound in RGP together with syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) to determine their biological activity in a cell model of oxidative stress. Syringic acid (SA) was the fourth major phenolic compound in RGP, and it was of interest over the third phenolic acid (protocatechuic acid (4,5-dihydroxybenzoic acid), because of its two methyl groups in C3 and C5 positions of the gallic acid (GA) molecule (Figure 1), therefore potentially adding interesting information on the effect of methoxylation on the biological properties of these phenolic compounds. Gastrointestinal digestion had minor effect on both acids, with a loss of only 3.6% of GA and up to 16% of SA (Table 9).

Study 2 in **Chapter 1** shows that physiologically relevant concentrations of the RGP phenolic extract and the selected phenolic acids, GA and SA were not cytotoxic for Caco-2 cells, not affecting endogenous antioxidant defences but decreasing the generation of reactive oxygen species (ROS), pointing to a direct antioxidant effect of these compounds. Surprisingly, this effect was more pronounced with SA (Figure 21), in spite of its dimethoxylated structure resulting in the loss of the ortho-diphenolic active structure (Bravo, 1998; Huang et al., 2005). However, when cells were submitted to an oxidative challenge induced by the pro-oxidant *t*-BOOH, pre-incubation with 1-10 µM concentrations of both phenolic compounds showed almost similar capacities preventing the oxidative damage, which were comparable to those of 1-10 µg/mL of RGPE, all the tested compounds limiting the cytotoxic effect of *t*-BOOH, the increase in ROS generation and protein oxidation, or the alteration of enzymatic and non-enzymatic endogenous defences. The similar outcomes with GA and SA suggest that methoxylation of hydroxybenzoic acids would have little effect on their biological activity as antioxidants in cell systems, although more assays testing different phenolic acids with different hydroxylation and methoxylation pattern would be necessary to confirm these findings.

Chemical characterization of green coffee beans and yerba mate extracts had already been performed by the research team (Baeza et al., submitted-a,c) that had also carried out acute human intervention trials to study the bioavailability and metabolism of their phenolic constituents, determining that reduced hydroxycinnamic acids derivatives originated by the colonic microbiota, DHCA and DHFA, were the major metabolites in plasma and urine (Gomez-Juaristi, 2015). In previous studies in HepG2 cells, green coffee and yerba mate extracts, as well as their major phenolic constituents (5-CQA and 3,5-DCQA as model molecules of mono- and dicaffeoylquinic acids, respectively) and metabolites (DHCA and DHFA) showed their capacity protecting these hepatic cells from the oxidative injury elicited by *t*-BOOH cells (Baeza et al., 2014, 2016). Since these hydroxycinnamic-rich beverages showed beneficial properties in cardiovascular disease, decreasing blood pressure and inflammatory markers in healthy and CVD-risk subjects, we wanted to study their potential effect at vascular level. Vascular cells produce ROS that may contribute to the endothelial dysfunction associated with vascular diseases; therefore, it was relevant to assess the effects of green coffee beans and yerba mate extracts and their phenolic constituents and metabolites on the antioxidant status of endothelial cells and their effect counteracting the pro-inflammatory challenge of the cytokine TNF-α, studies addressed in **Chapter 2**.

Direct treatment of EA.hy926 human umbilical cord endothelial cells with GCBE and YME, 5-CQA and the two metabolites DHCA and DHFA was not cytotoxic to these cells and did not modify antioxidant defences or levels of biomarkers of oxidative stress (Table 15); however, it significantly decreased ROS production in un-challenged cells (Figure 27), showing their antioxidant effect in this cell system, although the highest concentration of YME (50 µg/mL) seemed to cause a pro-oxidant response, as it has also been referred for high doses of phenolic antioxidants (Bravo, 1998; Huang et al., 2005). This regulation of ROS levels when cells were incubated with the test compounds point to a better competence of cells to face an oxidant challenge. This was indeed confirmed by the results of the protective experiments, where cells pre-incubated with the phenolic extracts, cholorogenic acid and metabolites responded better to the pro-inflammatory, oxidative challenge elicited by incubation with TNF-α (Figures 28-36). Moreover, improvement in eNOS levels in the challenged cells show a potential anti-inflammatory activity of these compounds and therefore a

relevant role against endothelial dysfunction. It is noteworthy the fact that microbial metabolites were very effective counteracting TNF-α-induced dysregulation of eNOS, even at 0.1 μM concentrations of DHCA and DHFA, showing better effect than 5-CQA or the coffee and mate extracts, pointing to the potent anti-inflammatory activity of these phenolic metabolites (Figure 36).

Finally, if green coffee and yerba mate were to be used as functional food ingredients or nutraceuticals, where higher consumption rates than when taken as part of normal dietary intakes are to be foreseen, it was important to ascertain their safety. More extensive studies at this respect would be necessary, but a preliminary screening of their potential effect inducing proliferation of malignant cells was contemplated as a key objective in view of the contradictory evidences from epidemiological observational studies on the association of yerba mate and coffee (i.e. roasted coffee) intake and increased risk or incidence of several types of cancer. Those that have been more frequently associated with coffee and mate consumption were selected for this preliminary study, namely lung, oesophageal and urinary bladder cancers. Also, one of the cancers with the highest mortality rates worldwide, colorectal cancer, was included in the study because of the potential impact of both foodstuffs in such prevalent malignancy, and also because it was a type of cancer where the effect of coffee and mate were in clear contradiction, as reviewed in **Chapter 3**.

For this study, human cancer cell lines representative of the different types of cancer mentioned above were used, namely colon adenocarcinoma (Caco-2), lung (A549), oesophageal (OE-33), and urinary bladder (T24) human carcinoma cells, as well as a normal, non-cancer colon cell line (CCD-18Co). The doses of GCBE and YME used, as those of the major mono- and dicaffeoylquinic acids (5-CQA and 3,5-DCQA), non-esterified caffeic and ferulic acids, the metabolites DHCA and DHFA, and the methylxanthine caffeine, were high, supraphysiological levels to cover potential high intakes of nutraceuticals containing these phenolic-rich products but also to test potential pharmacological effects. Yerba mate showed to be a very potent anti-proliferative agent, decreasing cell viability and proliferation of the four cancer cell lines even at physiologically relevant doses (10 μg/mL). Although it also had antiproliferative effects in the non-cancer cell line, lower doses not only didn't show cytotoxic action in these normal cells, but even had protective effects, also reducing levels of reactive oxygen species (Figures 38 and 39).

GCBE, in spite of its phenolic composition similar to that of yerba mate, was less efficient than the mate extract, which might be accounted for the additional presence of flavonols in mate, with important antiproliferative action (Ramos, 2008), and for its higher content in DCQAs (Baeza et al., submitted-b), DCQAs (3,4-, 3,5- and 4,5-DCQA) having shown to dose-dependently inhibit cancer cell proliferation, inducing apoptosis mediated by caspase 3 activation and expression of c-Jun (Jurata et al., 2007).

. The hydroxycinnamic acids 5-CQA and 3,5-DCQA were more efficient reducing cell viability and/or proliferation than the non-esterified caffeic acid (Tables 17 and 18), although ferulic acid showed a higher antiproliferative action compared to caffeic acid and the CQAs, reducing proliferation of all cancer cell lines even at the lowest dose tested (10 μM). In contrast, the reduced molecule, DHFA, was less active than FA and DHCA (Table 19). The number of caffeoyl groups has been suggested to be a key factor regulating the growth inhibitory activity of hydroxycinnamoylquinic esters and the antimutagenic capacity of CQA derivatives (Yashimoto et al., 2002; Iwai et al., 2004), in agreement with the higher activity of 3,5-DCQA vs. 5-CQA observed in the present work. Also, methoxylation of the caffeoyl group at the C3 position has been associated with lower antiproliferative activity against cancer cells (Iwai et al., 2004), which is in contrast with our findings since ferulic acid was clearly superior to caffeic acid reducing cell proliferation. The results with the reduced microbial metabolites DHCA and DHFA, however, are in line with the proposed lower activity of C3-methylated cinnamoyl acids, since the methoxylated DHFA was in general less effective than DHCA reducing cell viability and proliferation.

In all, green coffee and especially yerba mate, and their CQA and DCQA derivatives would not be expected to increase proliferation of cancer cells, in turn negatively affecting cell viability and proliferation in a dose- and time-dependent manner, being physiological doses non-cytotoxic to cancer and normal, non-cancer cells, even reducing basal ROS production in line with the antioxidant capacity seen in HepG2 (Baeza et al., 2014, 2016) and EA.hy926 cells (Chapter 2). Therefore, yerba mate might have interesting anti-proliferative potential in cancer prevention. However, these are preliminary studies that require further in-depth investigation to confirm this relationship and to determine the subjacent mechanisms of action.

It is especially relevant the outcomes related to the biological properties of phenolic metabolites. Caffeic and ferulic acids can be found as free molecules in different foods, although they are also metabolites resulting from de-esterification of caffeoylquinic and feruloylquinic acids, respectively. Ferulic acid can also be a phase II metabolite of caffeic acid after methylation by the catechol-*O*-methyl transferase (COMT) enzyme. As just mentioned, ferulic acid was a more potent antiproliferative molecule than caffeic acid, 5-CQA or 3,5-DCQA (Tables 17 and 18).

In line with this, microbial metabolites DHFA and particularly DHCA showed remarkable antioxidant and anti-inflammatory effects on EA.hy926 endothelial cells (**Chapter 2**), as well as antiproliferative actions against the studied cancer cell lines (Table 19), DHCA also showing cytoprotective and antioxidant effects reducing ROS generation in CCD-18Co normal colon cells (Figure 38). In agreement, Baeza et al. (2016) showed that DHCA had similar protective effects to those of YME and mono- and dicaffeoylquinic acids in HepG2 cells preventing *t*-BOOH-induced oxidative stress. Also, Fernandez-Millan et al. (2014) showed that microbial phenolic metabolites improved pancreatic beta-cell function and protected these cells from oxidative stress via ERKs and PKC pathways.

These findings are of great relevance, confirming the biological activity of phenolic metabolites. Many authors have shed doubts on the role of polyphenolic compounds in disease due to the limited bioavailability of most (poly)phenols and the extensive metabolism of the absorbed compounds. In fact, the biological activity of many polyphenols, notably that of flavonoids, is drastically reduced after phase II metabolism (methylation, glucuronidation and/or sulphation) (Manach et al., 2005; Williamson and Manach, 2005). Some authors, however, have hypothesized on the intracellular de-conjugation of phenolic metabolites in target tissues (Pérez-Vizcaíno et al., 2012), since molecules not found in plasma after oral administration (e.g. quercetin) had biologically demonstrable systemic effects at vascular level (vasodilator and anti-hypertensive actions) in spite of the circulating conjugated metabolites having weak activity *in vitro*. Moreover, non-absorbed polyphenols can be metabolized by the colonic microflora, yielding metabolites that are absorbed by the colonic mucosa and distributed to different tissues and organs. As seen here, those metabolites preserve or even potentiate

the biological activity of the parent phenolic compounds, having potent antioxidant, anti-inflammatory and anti-proliferative effects.

The results of the present work put forward the beneficial biological properties of phenolic-rich compounds such as red grape pomace, green coffee beans and yerba mate as seen in different cell models of oxidative stress, vascular function and cancer. Those biological properties of the phenolic extract of the studied foodstuffs, their major phenolic constituents and metabolites suggest beneficial effects in different pathologies (Figure 42) and sustain their use as functional foods ingredients or nutraceuticals.

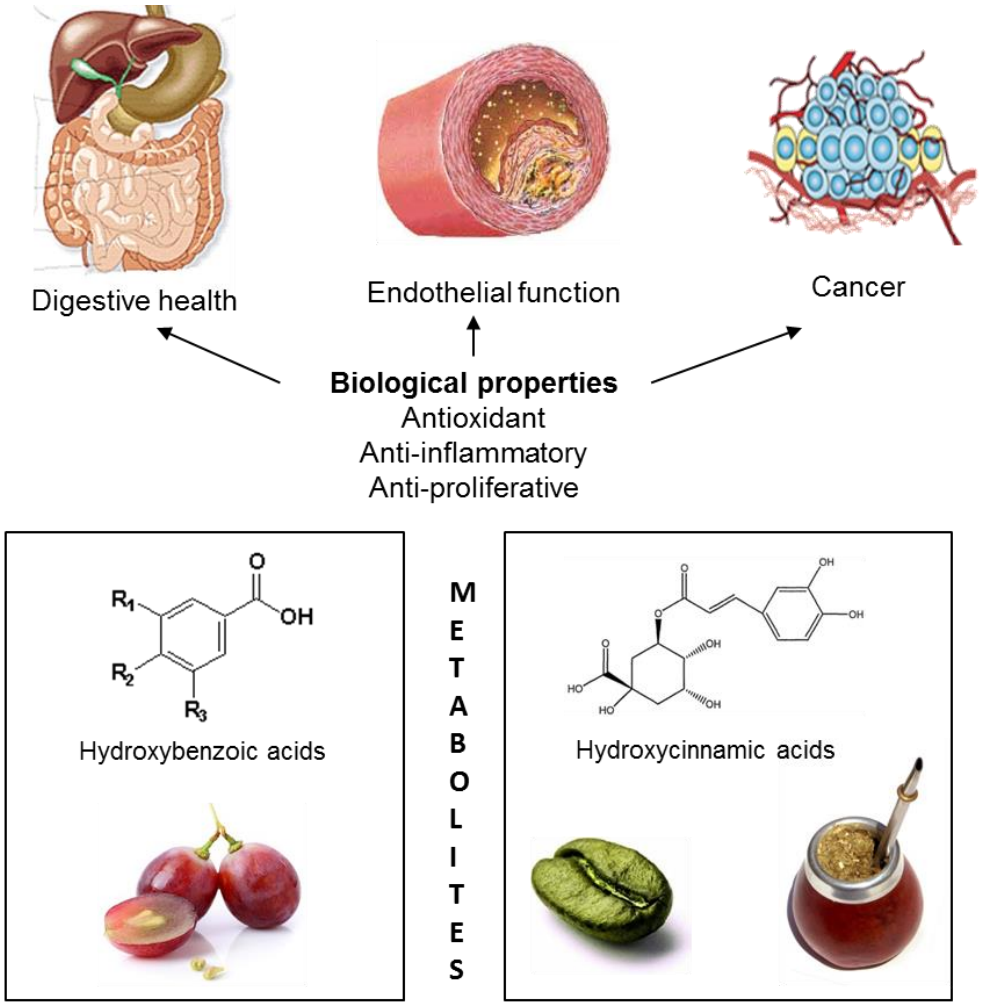


Figure 42. Biological properties and health implications of phenolic antioxidants and metabolites in red grape pomace, green coffee beans and yerba mate.

Conclusions

Conclusions

1. The red grape pomace phenolic extract is rich in hydroxybenzoic acids, flavonols and hydroxycinnamic acids, with high antioxidant capacity against $ABTS^{\bullet+}$ and peroxyl radicals, and reducing power.

2. Red grape pomace was microbiologically safe during 9 months storage under controlled temperature conditions. However, storage periods under 6 months are advisable. Although the phenolic composition and antioxidant capacity were stable during 6 months storage at 4 and 25 °C, longer times (9 months) result in colour changes associated to increased condensed tannins content and a remarkable loss of antioxidant capacity.

3. Gastrointestinal digestion can modify the biological properties of grape pomace since, although gastric digestion did not modify the grape by-product, during intestinal digestion the antioxidant capacity and the phenolic content of the pomace decreased significantly.

4. Treatment of colonic Caco-2 cells with physiological doses of red grape pomace phenolic extract, and gallic and syringic acids did not affect cell viability, antioxidant defenses or protein oxidation, these products proved their efficiency protecting cells against oxidative stress induced by the pro-oxidant *tert*-butylhydroperoxide.

5. Treatment of human umbilical endothelial cells EA.hy926 with the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) increased oxidative stress and the levels of endothelial nitric oxide synthase (eNOS) as markers of endothelial dysfunction. However, physiological doses of phenolic extracts of green coffee and yerba mate, their main phenolic constituent, 5-caffeoylquinic acid, and the microbial metabolite dihydrocaffeic acid partially prevented the inflammatory damaged induced by TNF-α.

6. Yerba mate and green coffee phenolic extracts and their phenolic constituents (5-caffeoylquinic and 3,5-dicaffeoylquinic acids) decreased viability and proliferation of different human cell lines of colon (Caco-2), lung (A549), oesophagus (OE-33), and urinary bladder (T24) cancer, not causing

cytotoxicity in a non-cancer human cell line (CCD-18Co). Metabolites ferulic, dihydrocaffeic and dihydroferulic acids were less efficient, having variable effects depending on the cell line and dose. It is especially relevant the antiproliferative effect of yerba mate, likely resulting from a synergic effect of its phenolic constituents.

7. Metabolites originated from the colonic microbiota, especially dihydrocaffeic acid, maintain their biological activity as shown by their efficiency protecting against TNF-α pro-inflammatory effect and their antiproliferative capacity in human cancer cells.

General conclusion

Red grape pomace, green coffee and yerba mate present relevant biological properties from a health point of view and thus great potential as functional food ingredients or nutraceuticals.

Conclusiones

Conclusiones

1. El extracto fenólico procedente del subproducto de uva tinta es rico en ácidos hidroxibenzoicos, flavonoles y acidos hidroxicinámicos, mostrando una importante capacidad antioxidante frente a los radicales $ABTS^{\bullet+}$ y peroxílicos, así como poder reductor.

2. El subproducto de uva es microbiológicamente seguro durante un periodo de conservación de 9 meses bajo condiciones controladas de temperatura. No obstante, se recomienda un periodo de almacenamiento no superior a 6 meses pues, si bien la composición fenólica y capacidad antioxidante del subproducto de uva tinta permanecen estables durante 6 meses de conservación a 4 y 25 °C, a tiempos más prolongados (9 meses) se produce un cambio de color asociado al aumento del contenido de taninos condensados junto a una notable disminución de la capacidad antioxidante.

3. La digestión gastrointestinal puede modificar las propiedades biológicas del subproducto de uva tinta pues, aunque la etapa gástrica no produjo cambios, durante la etapa intestinal disminuyeron significativamente la capacidad antioxidante y el contenido polifenólico.

4. El tratamiento de células colónicas Caco-2 con dosis fisiológicas del extracto del subproducto de uva tinta y los ácidos gálico y siríngico no alteró la viabilidad celular, las defensas antioxidantes o la oxidación proteica, mostrando su efectividad protegiendo frente al estrés oxidativo inducido por el pro-oxidante *tert*-butilhidroperóxido.

5. El tratamiento de células endoteliales humanas de cordón umbilical EA.hy926 con la citoquina pro-inflamatoria factor de necrosis tumoral alfa (TNF-α) produjo un aumento del estrés oxidativo y de los niveles del enzima óxido nítrico sintasa endotelial (eNOS), indicativos de disfunción endotelial. Sin embargo, dosis fisiológicas de extractos fenólicos de café verde y yerba mate, de su compuesto fenólico mayoritario, el ácido 5-cafeoilquínico, y del metabolito microbiano ácido dihidrocafeico previenen parcialmente el daño inflamatorio inducido por TNF-α.

6. Los extractos de yerba mate y café verde, así como sus principales constituyentes fenólicos (ácidos 5-cafeoilquínico y 3,5-dicafeoilquínico), disminuyeron la viabilidad y proliferación de distintas líneas humanas de cáncer de colon (células Caco-2), pulmón (A549), esófago (OE-33) y vejiga urinaria (T24), sin ser citotóxicos en una línea celular no cancerosa (células CCD-18Co). Los metabolitos ácidos ferúlico, dihidroferúlico y dihidrocafeico presentaron efectos menores y variables dependiendo de la línea celular y la dosis empleada. Es especialmente destacable el efecto antiproliferativo de yerba mate, posiblemente derivado del efecto sinérgico de sus constituyentes fenólicos.

7. Los metabolitos de origen microbiano, en especial el ácido dihidrocafeico, mantienen su actividad biológica, demostrada por su efectividad protegiendo frente a la actividad pro-inflamatoria del TNF-α y por su capacidad antiproliferativa frente a células cancerígenas humanas.

Conclusión general

Los subproductos de uva tinta, el café verde y la yerba mate presentan propiedades biológicas relevantes desde el punto de vista de la salud y, por tanto, gran potencial como ingredientes de alimentos funcionales o de nutracéuticos.

References

References

Actis-Goretta, L., Mackenzie, G.G., Oteiza, P.I., Fraga, C.G., 2002. Comparative study on the antioxidant capacity of wines and other plant-derived beverages. Annals of the New York Academy of Sciences 957, 279-283.

Aggarwal, B.B., Shishodia, S., 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochemical Pharmacology 71, 1397-1421.

Aizpurua-Olaizola, O., Navarro, P., Vallejo, A., Olivares, M., Etxebarria, N., Usobiaga, A., 2016. Microencapsulation and storage stability of polyphenols from *Vitis vinifera* grape wastes. Food Chemistry 190, 614-621.

Akhtar, S., Musthapa, S., Sharma, S., Katiyar, S., 2007. Dietary grape seed proanthocyanidins suppress UVB-induced oxidative stress and activation of MAPK proteins and nuclear factor-κB through restoration of MAP kinase phosphatases in SKH-1 hairless mice. Cancer Research 67, 2490-2490.

Akter, S., Kashino, I., Mizoue, T., Matsuo, K., Ito, H., Wakai, K., Nagata, C., Nakayama, T., Sadakane, A., Tanaka, K., Tamakoshi, A., Sugawara, A., Sawada, N., Inoue, M., Tsugane, S., Saazuki, S., 2016. Coffee drinking and colorectal cancer risk: an evaluation based on a systematic review and meta-analysis among the Japanese population. Japanese Journal of Clinical Oncology 46, 781-787.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2008. Molecular Biology of the Cell. New York: Garland Science; Classic Textbook 5th Edition. Chapter 20 Cancer, 1205-1268.

Alia, M., Mateos, R., Ramos, S., Lecumberri, E., Bravo, L., Goya, L., 2006a. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). European Journal of Nutrition 45, 19-28.

Alia, M., Ramos, S., Mateos, R., Bravo, L., Goya, L., 2005. Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). Journal of Biochemical and Molecular Toxicology 19, 119-128.

Alia, M., Ramos, S., Mateos, R., Granado-Serrano, A. B., Bravo, L., Goya, L., 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. Toxicology and Applied Pharmacology 212, 110-118.

Alonso-Salces, R.M., Guillou, C., Berrueta, L.A., 2009b. Liquid chromatography coupled with ultraviolet absorbance detection, electrospray ionization, collision-induced dissociation and tandem mass spectrometry on a triple quadrupole for the on-line characterization of polyphenols and methylxanthines in green coffee beans. Rapid Communications in Mass Spectrometry 23, 363-383.

Alonso-Salces, R.M., Serra, F., Reniero, F., Heberger, K., 2009a. Botanical and geographical characterization of green coffee (*Coffea arabica* and *Coffea canephora*): chemometric evaluation of phenolic and methylxanthine contents. Journal of Agricultural and Food Chemistry 57, 4224-4235.

Amigo-Benavent, M., Clemente, A., Caira, S., Stiuso, P., Ferranti, P., del Castillo, M.D., 2014. Use of phytochemomics to evaluate the bioavailability and bioactivity of antioxidant peptides of soybean β-conglycinin. Electrophoresis 35, 1582-1589.

Andersen, L.F., Jacobs, D.R.Jr., Carlsen, M.H., Blomhoff R., 2006. Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. The American Journal of Clinical Nutrition 83, 1039-1046.

Andersen, T., Fogh, J., 2001. Weight loss and delayed gastric emptying following a South American herbal preparation in overweight patients. Journal of Human Nutrition and Dietetics 14, 243-250.

Andrici, J., Eslick, G.D., 2013. Mate consumption and the risk of esophageal squamous cell carcinoma: a meta-analysis. Diseases of the Esophagus 26, 807-816.

Anesini, C., Ferraro, G., Filip, R., 2006. Peroxidase-like activity of *Ilex paraguariensis.* Food Chemistry 97, 459-464.

Anhê, F.F., Desjardins, Y., Pilon, G., Dudonné, S., Genovese, M.I., Lajolo, F.M., Marette, A., 2013. Polyphenols and type 2 diabetes: A prospective review. Pharma Nutrition 1, 105-114.

Anter, E., Chen, K., Shapira, O. M., Karas, R. H., Keaney, J. F., 2005. mitogen-activated protein kinase activates eNOS in endothelial cells by an estrogen receptor α-dependent pathway in response to black tea polyphenols. Circulation research 96, 1072-1078.

Antoniolli A, Fontana A.R., Piccoli, P., Bottini, R., 2015. Characterization of polyphenols and evaluation of antioxidant capacity in grape pomace of the cv. Malbec. Food Chemistry 178, 172-178.

AOAC (1999) Official Methods of Analysis of AOAC International. 16th Edition, 5th Revision. AOAC International, Arlington, VA.

Appeldoorn, M.M., Venema, D.P., Peters, T.H., Koenen, M.E., Arts, I.C., Vincken, J.P., Gruppen, H., Keijer, J., Hollman, P.C., 2009. Some phenolic compounds increase the nitric oxide level in endothelial cells in vitro. Journal of Agricultural and Food Chemistry 57, 7693-7699.

Arapitsas, P., Corte, A.D., Gika, H., Narduzzi, L., Mattivi, F., Theodoridis, G., 2016. Studying the effect of storage conditions on the metabolite content of red wine using HPLC LC-MS based metabolomics. Food Chemistry 197, 1331-1340.

Araujo, J.R., Gonçalves, P., Martel, F., 2011. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. Nutrition Research 31, 77-87.

Arcari, D.P., Bartchewsky, W., Jr., dos Santos, T.W., Oliveira, K.A., DeOliveira, C.C., Gotardo, E.M., Pedrazzoli, J., Jr., Gambero, A., Ferraz, L. F., Carvalho Pde, O., Ribeiro, M.L., 2011. Anti-inflammatory effects of yerba mate extract (*Ilex paraguariensis*) ameliorate insulin resistance in mice with high fat diet-induced obesity. Molecular and Cellular Endocrinology *335*, 110-115.

Arcari, D.P., Bartchewsky, W., dos Santos, T.W., Oliveira, K.A., Funck, A., Pedrazzoli, J., de Souza, M.F., Saad, M.J., Bastos, D.H., Gambero, A., Carvalho Pde, O., Ribeiro, M.L., 2009. Antiobesity effects of yerba mate extract (*Ilex paraguariensis*) in high-fat diet-induced obese mice. Obesity (Silver Spring, Md.) 17, 2127-2133.

Arcari, D.P., Santos, J.C., Gambero, A., Ribeiro, M.L., 2013. The in vitro and in vivo effects of yerba mate (*Ilex paraguariensis*) extract on adipogenesis. Food Chemistry 141, 809-815.

Arts, I.C., Hollman, P.C., 2005. Polyphenols and disease risk in epidemiologic studies. The American Journal of Clinical Nutrition 81, 317S-325S.

Arts, I.C., van de Putte, B., Hollman, P.C., 2000a. Catechin contents of foods commonly consumed in The Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. Journal of Agricultural and Food Chemistry 48, 1746-1751.

Arts, I.C., van de Putte, B., Hollman, P.C., 2000b. Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk. Journal of Agricultural and Food Chemistry 48, 1752-1757.

Artursson, P., Karlsson, J., 1991. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochemical and Biophysical Research Communications 175, 880-885.

Baenas, N., Silván, J.M., Medina, S., de Pascual-Teresa, S., García-Viguera, C., Moreno, D.A., 2015. Metabolism and antiproliferative effects of sulforaphane and broccoli sprouts in human intestinal (Caco-2) and hepatic (HepG2) cells. Phytochemistry Reviews 14, 1035-1044.

Baeza, G., Amigo-Benavent, M., Sarriá, B., Goya, L., Mateos, R., Bravo, L., 2014. Green coffee hydroxycinnamic acids but not caffeine protect human HepG2 cells against oxidative stress. Food Research International 62, 1038-1046.

Baeza, G., Sarriá, B., Mateos, R., Bravo, L., 2016. Dihydrocaffeic acid, a major microbial metabolite of chlorogenic acids, shows similar protective effect than a yerba mate phenolic extract against oxidative stress in HepG2 cells. Food Research International 87, 25-33.

Baeza, G., Sarriá, B., Mateos, R., Bravo, L., LC-MS^n characterization of saponins in mate (*Ilex paraguariensis*) and their quantification by HPLC-DAD. Food Chemistry, submitted.

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., Joshi, S.S., Pruess, H.G., 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 148, 187-197.

Barringer, T. A., Hatcher, L., Sasser, H. C., 2011. Potential benefits on impairment of endothelial function after a high-fat meal of 4 weeks of flavonoid supplementation. Evidence-Based Complementary and Alternative Medicine 2011, 1-6.

Bates, M.N., Hopenhayn, C., Rey, O.A., Moore, L.E., 2007. Bladder cancer and mate consumption in Argentina: a case-control study. Cancer Letter 246, 268-273.

Benetou, V., Orfanos, P., Lagiou, P., Trichopoulos, D., Boffetta, P., Trichopoulou, A., 2008. Vegetables and fruits in relation to cancer risk: evidence from the Greek EPIC cohort study. Cancer Epidemiology, Biomarkers & Prevention 17, 387-392.

Benzie, I.F., Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Analytical Biochemistry 239, 70-76.

Bermudez-Soto, M., Tomas-Barberan, F., Garcia-Conesa, M.T., 2007. Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion. Food Chemistry 102, 865-874.

Bhoo-Pathy, N., Peeters, P.HM., Uiterwaal, C. SPM., Bueno-de-Mesquita, H. B., Bulgiba, A. M., Bech, B. H., Overvad, K., Tjonneland, A., Olsen, A., Clavel-Chapelon, F., Fagherazzi, G., Perquier, F., Teucher, B., Kaaks, R., Schutze, M., Boeing, H., Lagiou, P., Orfanos, P., Trichopoulou, A., Agnoli, C., mattiello, A., Palli, D., Tumino, R., Sacerdote, C., Van Duijnhoven, F. J.B., Braaten, T., Lund, E., Skeie, G., Redondo, M.-L., Buckland, G., Perez, M.J.S., Chirlaque, M.-D., Ardanaz, E., Amiano, P., Wirfalt, E., Wallstrom, P., Johansson, I., Nilsson, L. M., Khaw, J-T., Wareham, N., Allen, N. E., Key, T. J. Rinaldi, S., Romieu, I., Gallo, V., Riboli, E., Van Gils, C. H., 2015. Coffee and tea consumption and risk of pre- and postmenopausal breast cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study. Breast Cancer Research 17, 1-12.

Bixbym, M., Spielerm, L., Meninim, T., Gugliuccim, A., 2005. *Ilex paraguariensis* extracts are potent inhibitors of nitrosative stress: A comparative study with green tea and wines using a protein nitration model and mammalian cell cytotoxicity. Life Sciences 77, 345-358.

Block, G., Patterson, B., Subar, A., 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutrition Cancer 18, 1-29.

Boaventura, B.C., Di Pietro, P.F., Stefanuto, A., Klein, G.A., de Morais, E.C., de Andrade, F., Wazlawik, E., & da Silva, E.L., 2012. Association of mate tea (*Ilex paraguariensis*) intake and dietary intervention and effects on oxidative stress biomarkers of dyslipidemic subjects. Nutrition 28, 657-664.

Bohn, S.K., Blomhoff, R., Paur, I., 2014. Coffee and cancer risk, epidemiological evidence, and molecular mechanisms. Molecular Nutrition & Food Research 58, 915-930.

Bonita, J.S., Mandarano M, Shuta D, Vinson J., 2007. Coffee and cardiovascular disease: in vitro, cellular, animal, and human studies. Pharmacological Research 55, 187-198.

Borges, M. C., Vinolo, M. A., Nakajima, K., de Castro, I. A., Bastos, D. H., Borelli, P., Fock, R. A., Tirapegui, J., Curi, R., Rogero, M.M., 2013. The effect of mate tea (*Ilex paraguariensis*) on metabolic and inflammatory parameters in high-fat diet-fed Wistar rats. International Journal of Food Sciences and Nutrition 64, 561-569.

Botterweck, A.A., van den Brandt, P.A., Goldbohm, R.A., 1998. A prospective cohort study on vegetable and fruit consumption and stomach cancer risk in The Netherlands. American Journal of Epidemiology 148, 842-853.

Box, J.D., 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Research 17, 511-525.

Bracescom, N., Dell, M., Rocha, A., Behtash, S., Menini, T., Gugliucci, A., Nunes, E., 2003. Antioxidant activity of a botanical extract preparation of *Ilex paraguariensis*: Prevention of DNA double-strand breaks in *Saccharomyces cerevisiae* and human low-density lipoprotein oxidation. The Journal of Alternative and Complementary Medicine 9, 379-387.

Bracesco, N., Sanchez, A.G., Contreras, V., Menini, T., Gugliucci, A., 2011. Recent advances on *Ilex paraguariensis* research: minireview. Journal of Ethnopharmacology 136, 378-384.

Bradamante, S, Barenghi, L, Villa, A., 2004. Cardiovascular protective effects of resveratrol. Cardiovascular Drug Reviews 22, 169-188.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews 56, 317-333.

Bravo, L., Goya, L., Lecumberri, E., 2007. LC/MS characterization of phenolic constituents of mate (*Ilex paraguariensis,* St. Hil.) and its antioxidant activity compared to commonly consumed beverages. Food Research International 40, 393-405.

Bravo, L., Mateos, R., Sarria, B., Baeza, G., Lecumberri, E., Ramos, S., Goya, L., 2014. Hypocholesterolaemic and antioxidant effects of yerba mate (*Ilex paraguariensis*) in high-cholesterol fed rats. Fitoterapia 92, 219-229.

Bravo, L., Saura-Calixto, F., 1998. Characterization of dietary fiber and the *in vitro* indigestible fraction of grape pomace. American Journal of Enology and Viticulture 49, 135-141.

Bruno, R.S., Dugan, C.E., Smyth, J.A., DiNatale, D.A., Koo, S.I., 2008. Green tea extract protects leptin-deficient, spontaneously obese mice from hepatic steatosis and injury. Journal of Nutrition 138, 323-331.

Calo, R., Marabini, L., 2014. Protective effect of *Vaccinium myrtillus* extract against UVA- and UVB-induced damage in a human keratinocyte cell line (HaCaT cells). Journal of Photochemistry and Photobiology B: Biology 132, 27-35.

Cantos, E., Espín, J.C., Tomás-Barberán, F.A., 2002. Varietal differences among the polyphenol rofiles of seven table grape cultivars studied by LC−DAD−MS−MS. Journal of Agricultural and Food Chemistry 50, 5691-5696.

Cárdenas, C., Quesada, A. R., Medina, M. A., 2014. Insights on the antitumor effects of kahweol on human breast cancer: decreased survival and increased production of reactive oxygen species and cytotoxicity. Biochemical and Biophysical Research Communications 447, 452-458.

Carluccio, M. A., Siculella, L., Ancora, M. A., Massaro, M., Scoditti, E., Storelli, C., Visioli, F., Distante, A., De Caterina, R., 2003. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties

of Mediterranean diet phytochemicals. Arteriosclerosis, Thrombosis, and Vascular Biology 23, 622-629.

Castellsague, X., Muñoz, N., De Stefani, E., Victora, C.G., Castelletto, R., Rolón, P.A., 2000. Influence of mate drinking, hot beverages and diet on esophageal cancer risk in South America. International Journal of Cancer 88, 658-664.

Chandra, S., Gonzalez de Mejia E., 2004. Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. Journal of Agricultural and Food Chemistry 52, 3583-3589.

Chang, W.C., Chen, C.H., Lee, M.F., Chang, T., Yu, Y.M., 2010. Chlorogenic acid attenuates adhesion molecules upregulation in IL-1 beta-treated endothelial cells. European Journal of Nutrition 49, 267-275.

Chen, C.H., Liu, T.Z., Chen, C.H., Wong, C.H., Chen, C.H., Lu, F.J., Chen, S.C., 2007. The efficacy of protective effects of tannic acid, gallic acid, ellagic acid, and propyl gallate against hydrogen peroxide-induced oxidative stress and DNA damages in IMR-90 cells. Molecular Nutrition & Food Research 51, 962-968.

Chen, D., Milacic, V., Chen, M.S., Wan, S.B., Lam, W.H., Huo, C., Landis-Piwowar, K.R., Cui, Q.C., Wali, A., Chan, T.H., Dou, Q.P., 2008. Tea polyphenols, their biological effects and potential molecular targets. Histology and Histopathology 23, 487-496.

Chen, J., Long, S., 2014. Tea and coffee consumption and risk of laryngeal cancer: a systematic review meta-analysis. PLoS ONE 9, e112006.

Chen, X., Chen Y., Bi, Y., Fu, N., Shan, C., Wang, S., Aslam, S., Wang, P., Xu, J., 2007. Preventive cardioprotection of erythropoietin against doxorubicin-induced cardiomyopathy. Cardiovascular Drugs and Therapy 21, 367-374.

Chen, X., Xun, K., Chen, L., Wang, Y., 2009. TNF-alpha, a potent lipid metabolism regulator. Cell Biochemistry and Function 27, 407-416.

Chiou, W.F., Shen, C.C., Chen, C.C., Lin, C.H., Huang,Y.L., 2009. Oligostilbenes from the roots of *Vitis thunbergii*. Planta Medica 75, 856-859.

Choi, S.-K., Zhang, X.-H., Seo, J.-S., 2012. Suppression of oxidative stress by grape seed supplementation in rats. Nutrition research and practice 6, 3-8.

Chouchouli, V., Kalogeropoulos, N., Konteles, S.J., Karvela, E., Makris, D.P., Karathanos, V.T., 2013. Fortification of yoghurts with grape (*Vitis vinifera*) seed extracts. LWT - Food Science and Technology 53, 522-529.

Chu, Y., Brown, P. H., Lyle, B. J., Chen, Y., Black, R. M., Williams, C. E., Lin, Y., Hsu, C., Cheng, I.H., 2009. Coffees cigh in lipophilic antioxidants and chlorogenic acid lactones are more neuroprotective than green coffees. Journal of Agricultural and Food Chemistry 57, 9801-9808.

Chuang, C.C., McIntosh, M.K., 2011. Potential mechanisms by which polyphenol-rich grapes prevent obesity-mediated inflammation and metabolic diseases. Annual Review of Nutrition 31, 155-176.

Chung, Y.-C., Huang, C.-C., Chen, C.-H., Chiang, H.-C., Chen, K.-B., Chen, Y.-J., Liu, C.-L., Chuang, L.-T., Liu, M., Hsu, C.-P., 2012. Grape-seed procyanidins inhibit the in vitro growth and invasion of pancreatic carcinoma cells. Pancreas 41, 447-454.

Clapp, B. R., Hingorani, A. D., Kharbanda, R. K., Mohamed-Ali, V., Stephens, J. W., Vallance, P., MacAllister, R. J., 2004. Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. Cardiovascular research 64, 172-178.

Clement, M.V., Hirpara, J.L., Chawdhury, S.H., Pervaiz, S., 1998. Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood 92, 996-1002.

Clemente, A., Moreno, F.J., Marin-Manzano M.C., Jimenez, E., Domoney, C., 2010. The cytotoxic effect of Bowman-Birk isoinhibitors, IBB1 and IBBD2, from soybean (*Glycine max*) on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases. Molecular Nutrition & Food Research 54, 396-405.

Clifford, M.N., Ramirez-Martinez, J.R., 1990. Chlorogenic acids and purine alkaloids contents of mate (*Ilex paraguariensis*) leaf and beverage. Food Chemistry 35, 13–21.

Corona, G., Deiana, M., Incani, A., Vauzour, D., Dessì M.A., Spencer J.P., 2007. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil

polyphenols underlies their anti-proliferative effects. Biochemical and Biophysical Research Communications 362, 606-611.

Crespy, V., Morand, C., Besson, C., Manach, C., Demigne, C., Remesy, C., 2002. Quercetin, but not its glycosides, is absorbed from the rat stomach. Journal of Agricultural and Food Chemistry 50, 618-621.

Ćurko, N., Kovačević Ganić, K., Gracin, L., Đapić, M., Jourdes, M., Teissedre, P.L., 2014. Characterization of seed and skin polyphenolic extracts of two red grape cultivars grown in Croatia and their sensory perception in a wine model medium. Food Chemistry 145, 15-22.

da Silva, E. R., da Silva, M. F., Fischer, H., Mortara, R. A., Mayer, M. G., Framesqui, K., Silber, A. M., Floeter-Winter, L. M., 2008. Biochemical and biophysical properties of a highly active recombinant arginase from Leishmania (*Leishmania*) amazonensis and subcellular localization of native enzyme. Molecular and Biochemical Parasitology 159, 104-111.

Daglia, M., Papetti, A., Gregotti, C., Bertè, F., Gazzani, G., 2000. In vitro antioxidant and ex vivo protective activities of green and roasted coffee. Journal of Agricultural and Food Chemistry 48, 1449-1454.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chimica Acta 329, 23-38.

D'Archivio, M., Filesi, C., Vari, R., Scazzocchio, B., Masella, R., 2010. Bioavailability of the polyphenols: status and controversies. International Journal of Molecular Science 11, 1321-1342.

Dasanayake, A.P., Silverman, A.J., Warnakulasuriya, S., 2010. Mate drinking and oral and oro-pharyngeal cancer: a systematic review and meta-analysis. Oral Oncology 46, 82-86.

Davalos, A., Fernandez-Hernando, C., Cerrato, F., Martinez-Botas, J., Gomez-Coronado, D., Gomez-Cordoves, C., Lasuncion, M.A., 2006. Red grape juice polyphenols alter cholesterol homeostasis and increase LDL-receptor activity in human cells in vitro. The Journal of Nutrition 136, 1766-1773.

De Andrade, F., de Albuquerque, C.A., Maraschin, M., da Silva, E.L., 2012. Safety assessment of yerba mate (*Ilex paraguariensis*) dried extract: results of acute and 90 days subchronic toxicity studies in rats and rabbits. Food and Chemical Toxicology 50, 328-334.

Décordé, K., Teissèdre, P.-L., Sutra, T., Ventura, E., Cristol, J.-P., Rouanet, J.-M., 2009. Chardonnay grape seed procyanidin extract supplementation prevents high-fat diet-induced obesity in hamsters by improving adipokine imbalance and oxidative stress markers. Molecular Nutrition & Food Research 53, 659-666.

Deiana, M., Loru, D., Incani, A., Rosa, A., Atzeri, A., Melis, M.P., Cabboi, B., Hollecker, L., Pinna, M.B., Argiolas, F., Murru, M., Dessi, M.A., 2012. Wine extracts from Sardinian grape varieties attenuate membrane oxidative damage in Caco-2 cell monolayers. Food Chemistry 134, 2105-2113.

De la Cerda-Carrasco, A., Lopez-Solis, R., Nunez-Kalasic, H., Pena-Neira, A., Obreque-Slier, E., 2015. Phenolic composition and antioxidant capacity of pomaces from four grape varieties (*Vitis vinifera* L.). Journal of the Science of Food and Agriculture 95, 1521-1527.

de Mejia, E.G., Song, Y.S., Heck, C.I., Ramirez-Mares, M., 2010. Yerba mate tea (*Ilex paraguraiensis*): phenolics, antioxidant capacity and in vitro inhibition of colon cancer cell proliferation. Journal of Functional Foods 2, 23-24.

de Moraes Pontilho Mph, P., Mariana Nunes da Costa Teixeira Mph, A., Yuan Bsc, C., Alves Luzia, L., Helena Markowicz Bastos, D., Helen Rondó, P., 2015. Yerba Mate (*Ilex Paraguariensis* A. St. Hil) and Risk Factors for Cardiovascular Diseases. Journal of Food and Nutrition Research 3, 182-190.

De Stefani, E., Boffetta, P., Deneo-Pellegrini, H., Correa, P., Ronco, A.L., Brennan, P., Ferro, G., Acosta, G., Mendilaharsu, M., 2007. Non-alcoholic beverages and risk of bladder cancer in Uruguay. BMC Cancer 7, 57-64.

De Stefani, E., Fierro, L., Correa, P., Fontham, E., Ronco, A., Larrinaga, M., Balbi, J., Mendilaharsu, M., 1996. Mate drinking and risk of lung cancer in males: a case-control study from Uruguay. Cancer Epidemiology, Biomarkers & Prevention 5, 515-519.

Delgado-Andrade, C., Morales, F.J., 2005a. Unraveling the contribution of melanoidins to the antioxidant activity of coffee brews. Journal of Agricultural and Food Chemistry 53,1403-1407.

Delgado-Andrade, C., Rufián-Henares, J.A., Morales, F.J., 2005b. Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. Journal of Agricultural and Food Chemistry 53, 7832-7836.

De Mejía, E.G., Song, Y.S., Heck, C.I., Ramírez-Mares, M., 2010. Yerba mate tea *(Ilex paraguariensis):* Phenolics, antioxidant capacity and in vitro inhibition of colon cancer cell proliferation. Journal of Functional Foods 2, 23-34.

De Morais, E.C., Stefanuto, A., Klein, G.A., Boaventura, B.C., de Andrade, F., Wazlawik, E., Di Pietro, P.F., Maraschin, M., da Silva, E.L., 2009. Consumption of yerba mate (*Ilex paraguariensis*) improves serum lipid parameters in healthy dyslipidemic subjects and provides an additional LDL-cholesterol reduction in individuals on statin therapy. Journal of Agricultural and Food Chemistry 57, 8316-8324.

Deng, Q., Penner, M.H., Zhao, Y., 2011. Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. Food Research International 44, 2712-2720.

De Stefani, E., Deneo-Pellegrini, H., Ronco, A.L., Boffetta, P., Brennan, P., Munoz, N., Castellsague, X., Correa, P., Mendilaharsu, M., 2003. Food groups and risk of squamous cell carcinoma of the oesophagus, a case–control study in Uruguay. British Journal of Cancer 89, 1209-1214.

Dhalla, N.S., Temsah, R.M., Netticadan, T., 2000. Role of oxidative stress in cardiovascular diseases. Journal of Hypertension 18, 655-673.

Di Lecce, G., Arranz, S., Jáuregui, O., Tresserra-Rimbau, A., Quifer-Rada, P., Lamuela-Raventós, R.M., 2014. Phenolic profiling of the skin, pulp and seeds of Albariño grapes using hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry. Food Chemistry 145, 874-882.

Ding, E. L., Hutfless, S. M., Ding, X., Girotra, S., 2006. Chocolate and prevention of cardiovascular disease: a systematic review. Nutrition & Metabolism 3, 2-13.

Ding, M., Bhupathiraju, S.N., Satija, A., van Dam, R.M., Hu, F.B., 2014. Long-term coffee consumption and risk of cardiovascular disease: a systematic review and a dose-response meta-analysis of prospective cohort studies. Circulation 129, 643-659.

Dohadwala, M.M., Vita, J.A., 2009. Grapes and cardiovascular disease. Journal of Nutrition 139, 1788S-1793S.

Duarte, G. S., Farah, A., 2011. Effect of simultaneous consumption ofmilk and coffee on chlorogenic acids bioavailability in humans. Journal of Agricultural and Food Chemistry 59, 7925-7931.

Durak, I., Cetin, R., Devrim, E., Erguder, I.B., 2005. Effects of black grape extract on activities of DNA turn-over enzymes in cancerous and non cancerous human colon tissues. Life Sciences 76, 2995-3000.

El Gharras, H., 2009. Polyphenols: food sources, properties and applications - a review. International Journal of Food Science and Technology 44, 2512-2518.

Ejaz, A., Wu, D., Kwan, P., Meydani, M., 2009. Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. The Journal of Nutrition 139, 919-925.

Emeis, J.J., Edgell, C.J., 1988. Fibrinolytic properties of a human endothelial hybrid cell line (Ea.hy 926). Blood 71, 1669-1675.

Esquivel, P., Jiménez, V.M., 2012. Functional properties of coffee and coffee by-products. Food Research International 46, 488-495.

European Commission, 2010. Functional Foods, Brussels, Belgium.

FAOSTAT, Food and Agriculture Organization of the United Nations. 2016. Available online: http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E (accessed on 2nd June, 2016).

Farah, A., Donangelo, C.M., 2006. Phenolic compounds in coffee. Brazilian Journal of Plant Physiology 18, 23-36.

Farah, A., Monteiro, M., Donangelo, C.M., Lafay, S., 2008. Chlorogenic acids from green coffee extract are bioavailable in humans. The Journal of Nutrition 138, 2309-2315.

Faried, A., Kurnia, D., Faried, L.S., Usman, N., Miyazaki, T., Kato, H., Kuwano, H., 2007. Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines. International Journal of Oncology 30, 605-613.

Feng, Y., McDonald, C., Vick, B., 1988. C-glycosylflavones from hard red spring wheat bran. Cereal Chemistry 65, 452-456.

Feng, R., Lu, Y., Bowman, L. L., Qian, Y., Castranova, V., Ding, M., 2005. Inhibition of activator protein-1, NF-κB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. Journal of Biological Chemistry 280, 27888-27895.

Fernandez-Gomez, B., Lezama, A., Amigo-Benavent, M., Ullate, M., Herrero, M., Martin, M.A., Mesa, M.D., del Castillo, M.D., 2016. Insights on the health benefits of the bioactive compounds of coffee silver skin extract. Journal of Functional Foods 25 197-207.

Fernandez-Millan, E., Cordero-Herrera, I., Ramos, S., Escriva, F., Alvarez, C., Goya, L., Martin, M.A., 2015. Cocoa-rich diet attenuates beta cell mass loss and function in young Zucker diabetic fatty rats by preventing oxidative stress and beta cell apoptosis. Molecular Nutrition & Food Research 59, 820-824.

Fernandez-Millan, E., Ramos, S., Alvarez, C., Bravo, L., Goya, L., Martin, M.A., 2014. Microbial phenolic metabolites improve glucose-stimulated insulin secretion and protect pancreatic beta cells against tert-butyl hydroperoxide-induced toxicity via ERKs and PKC pathways. Food and Chemical Toxicology 66, 245-253.

Ferreira, F., Vázquez, A., Güntner, C., Moyna, P., 1997. Inhibition of the passive diffusion of cholic acid by the *Ilex paraguariensis* St. Hil. saponins Phytotherapy Research 11, 79-81.

Ferruzzi, M.G., Lobo, J.K., Janle E.M., Cooper B., Simon, J.E., Wu Q.L., Welch, C., Ho, L., Weaver, C., Pasometti, G.M., 2009. Bioavailability of gallic acid and catechins from grape seed polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease. Journal of Alzheimer's Disease 18, 113-124.

Filip, R., Lotito, S.B., Ferraro, G., Fraga, C.G., 2000. Antioxidant activity of Ilex paraguariensis and related species. Nutrition Research 20, 1437-1446.

Filip, R., López, P., Giberti, G., Coussio, J., Ferraro G., 2001. Phenolic compounds in seven South American *Ilex* species. Fitoterapia 72, 774-778.

Fonseca, C.A., Otto, S.S., Paumgartten, F.J., Leitão, A.C., 2000. Nontoxic, mutagenic, and clastogenic activities of Mate-Chimarrao (*Ilex paraguariensis*). Journal of Environmental Pathology, Toxicology and Oncology 19, 333-346.

Fontana, A.R., Antoniolli, A., Bottini, R., 2013. Grape pomace as a sustainable source of bioactive compounds: Extraction, characterization, and biotechnological applications of phenolics. Journal of Agricultural and Food Chemistry 61, 8987–9003.

Ford, S., Obeidy, P., Lovejoy, D., Bedford, M., Nichols, L., Chadwick, C., Tucker, O., Lui, G., Kalinowski, D., Jansson, P., 2013. Deferasirox (ICL670A) effectively inhibits oesophageal cancer growth in vitro and in vivo. British Journal of Pharmacology 168, 1316-1328.

Forester, S.C., Waterhouse, A.L., 2010. Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2, 4, 6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells. Journal of Agricultural and Food Chemistry 58, 5320-5327.

Franceschi, S., Parpinel, M., La Vecchia, C., Favero, A., Talamini, R., Negri, E., 1998. Role of different types of vegetables and fruit in the prevention of cancer of the colon, rectum, and breast. Epidemiology 9, 338-341.

Frankel, E.N., Waterhouse, A.L., Kinsella, J.E., 1993. Inhibition of human LDL oxidation by resveratrol. The Lancet 341, 1103-1104.

Fukumura, D., Kashiwagi, S., Jain, R.K., 2006. The role of nitric oxide in tumour progression. Nature Review Cancer 6, 521-534.

Gallardo, E., Sarriá, B., Espartero, J.L., Gonzalez-Correa, J.A., Bravo, L., Mateos, R., 2016. Evaluation of the bioavailability and metabolism of nitroderivatives of hydroxytyrosol using Caco-2 and HepG2 human cell models. Journal of Agriculture and Food Chemistry 64, 2289-2297.

Galvano, F., La Fauci, L., Vitaglione, P., Fogliano, V., Vanella, L., Felgines, C., 2007. Bioavailability, antioxidant and biological properties of the natural free-

radical scavengers cyanidin and related glycosides. Annali dell'Istituto Superiore di Sanità 43, 382-393.

Gan, Y., Wu, J., Zhang, S., Li, L., Cao, S., Mkandawire, N., Ji, N., Herath, C., Gao, C., Xu, H., Zhou, Y., Song, X., Chen, S., Chen, Y., Yang, T., Li, J., Qiao, Y., Hu, S., Yin, Z., Lu, Z., 2016. Association of coffee consumption with risk of colorectal cancer: a meta-analysis of prospective cohort studies. Oncotarget, DOI 10.18632/oncotarget.8627.

Gao, H., Liu, Z., Qu, X., Zhao, Y., 2013a. Effects of Yerba Mate tea (*Ilex paraguariensis*) on vascular endothelial function and liver lipoprotein receptor gene expression in hyperlipidemic rats. Fitoterapia 84, 264-272.

Gao, H., Liu, Z., Wan, W., Qu, X., Chen, M., 2013b. Aqueous extract of Yerba Mate tea lowers atherosclerotic risk factors in a rat hyperlipidemia model. Phytotherapy Research 27, 1225-1231.

García-Alonso, F., Periago, M., Vidal-Guevara, M., Cantos, E., Ros, G., Ferreres, R., Abellán, P., 2003. Assessment of the antioxidant properties during storage of a dessert made from grape, cherry, and berries. Journal of Food Science 68, 1525-1530.

Garcia-Lafuente, A., Guillamon, E., Villares, A., Rostagno, M.A., Martinez, J.A., 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflammation Research 58, 537-552.

García-Lomillo, J., González-SanJosé, M.L., Del Pino-García, R., Rivero-Pérez, M.D., Muñiz-Rodríguez, P., 2014. Antioxidant and antimicrobial properties of wine byproducts and their potential uses in the food industry. Journal of Agricultural and Food Chemistry 62, 12595-12602.

Garcia-Viguera, C., Zafrilla, P., Romero, .P, Abellan, P., Artes, F., Tomas-Barberan, F.A., 1999. Color stability of strawberry jam as affected by cultivar and storage temperature. Journal of Food Science 64, 243-247.

Garrido, J., Borges, F., 2013. Wine and grape polyphenols - A chemical perspective. Food Research International 54, 1844–1858.

Gavrieli, A., Yannakoulia, M., Fragopoulou, E., Margaritopoulos, D., Chamberland, J.P., Kaisari, P., Kavouras, S.A., & Mantzoros, C.S., 2011.

Caffeinated coffee does not acutely affect energy intake, appetite, or inflammation but prevents serum cortisol concentrations from falling in healthy men. The Journal of Nutrition 141, 703-707.

Georgiev, V., Ananga, A., Tsolova, V., 2014. Recent advances and uses of grape flavonoids as nutraceuticals. Nutrients 6, 391-415.

Gil-Izquierdo, A., Zafrilla, P., Tomás-Barberán, F.A., 2002. An in vitro method to simulate phenolic compound release from the food matrix in the gastrointestinal tract. European Food Research and Technology 214, 155-159.

Giovannucci, E., 1998. Meta-analysis of coffee consumption and risk of colorectal cancer. American Journal of Epidemiology 147, 1043-1052.

Giovannelli, L., Testa, G., De Filippo, C., Cheynier, V., Clifford, M. N., Dolara, P., 2000. Effect of complex polyphenols and tannins from red wine on DNA oxidative damage of rat colon mucosa in vivo. European Journal of Nutrition 39, 207-212.

Glei, M., Kirmse, A., Habermann, N., Persin, C., Pool-Zobel, B.L., 2006. Bread enriched with green coffee extract has chemoprotective and antigenotoxic activities in human cells. Nutrition and Cancer 56,182-192.

Gnoatto, S.C.B., Schenkel, E.P., Bassani V.L., 2005. HPLC method to assay total saponins in Ilex paraguariensis aqueous extract. Journal of the Brazilian Chemical Society 16,1678-4790.

Goldenberg, D., 2002. Mate: a risk factor for oral and oropharyngeal cancer. Oral Oncology 38, 646-649.

Goldenberg, D., Golz, A., Joachims, H.Z., 2003. The beverage mate: a risk factor for cancer of the head and neck. Head and Neck 25, 595-601.

Gomez-Juaristi, M., 2015. Metabolismo de flavonoids y ácidos hidroxicinámicos de la dieta. Estudios de transporte *in vitro* y de biodisponibilidad en humanos. Doctoral Thesis, Universidad Complutense de Madrid, Spain.

Gonthier, M. P., Verny, M. A., Besson, C., Rémésy, C., Scalbert, A., 2003. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. The Journal of Nutrition 133, 1853-1859.

Gonzalez de Mejia, E., Song, Y.S., Ramirez-Mares, M.V., Kobayashi, H., 2005. Effect of yerba mate (*Ilex paraguariensis*) tea on topoisomerase inhibition and oral carcinoma cell proliferation. Journal of Agricultural and Food Chemistry 53, 1966-1973.

Gorzalczany, S., Filip, R., Alonso, M.R., Miño, J., Ferraro, G.E., Acevedo, C., 2001. Choleretic effect and intestinal propulsion of 'mate' (*Ilex paraguariensis*) and its substitutes or adulterants. Journal of Ethnopharmacology 75, 291-294.

Goya, L., Martín, M.A., Ramos, S., Mateos, R., Bravo, L., 2009. A cell culture model for the assessment of the chemopreventive potential of dietary compounds. Current Nutrition & Food Science 5, 56-64.

Goya, L., Mateos, R., Bravo, L., 2007. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. European Journal of Nutrition 46, 70-78.

Granado-Serrano, A.B., Martin, M.A., Bravo, L., Goya, L., Ramos, S., 2008. Time-course regulation of quercetin on cell survival/proliferation pathways in human hepatoma cells. Molecular Nutrition & Food Research 52, 457-464.

Granado-Serrano, A.B., Martin, M.A., Bravo, L., Goya, L., Ramos, S., 2010a. Quercetin modulates NF-κB and AP-1/JNK pathways to induce cell death in human hepatoma cells. Nutrition and Cancer 62, 390-401.

Granado-Serrano, A.B., Martín, M.Á., Bravo, L., Goya, L., Ramos, S., 2012. Quercetin Attenuates TNF-Induced Inflammation in Hepatic Cells by Inhibiting the NF-κB Pathway. Nutrition and Cancer 64, 588-598.

Granado-Serrano, A.B., Martin, M.A., Haegeman, G., Goya, L., Bravo, L., Ramos, S., 2010b. Epicatechin induces NF-κB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells. British Journal of Nutrition 103, 168-179.

Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos, S., 2006. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2 and inhibition of PI-3-kinase/AKT and ERK pathways in a human hepatoma cell line (HepG2). The Journal of Nutrition 136, 2715-2721.

Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos, S., 2009. A diet rich in cocoa attenuates N-nitrosodiethylamine-induced liver injury. Food and Chemichal Toxicology 47, 2499-2506.

Granado-Serrano, A.B., Martín, M.A., Izquierdo-Pulido, M., Goya, L., Bravo, L., Ramos, S., 2007. Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. Journal of Agricultural and Food Chemistry 55, 2020-2027.

Greenberg, J.A., Boozer, C.N., Geliebter, A., 2006. Coffee, diabetes, and weight control. The American Journal of Clinical Nutrition 84, 682-693.

Grigioni, G., Carduza, F., Irurueta, M., Pensel, N., 2004. Flavour characteristics of *Ilex paraguariensis* infusion, a typical Argentine product, assessed by sensory evaluation and electronic nose. Journal of the Science of Food and Agriculture 84, 427-432.

Guercio, B.J., Sato, K., Niedzwiecki, D., Ye, X., Saltz, L.B., Mayer, R.J., Mowat, R.B., Whittom, R., Hantel, A., Benson, A., Atienza, D., Messino, M., Kindler, H., Venook, A., Hu, F.B., Ogino, S., Wu, K., Willett, W.C., Giovannucci, E.L., Meyerhardt, J.A., Fuchs, C.S., 2015. Coffee intake, recurrence, and mortality in stage III colon cancer: results from CALGB 89803 (Alliance). Journal of Clinical Oncology 33, 3598-3607.

Gugliucci, A., 1996. Antioxidant effects of *Ilex paraguariensis*: induction of decreased oxidability of human LDL in vivo. Biochemical and Biophysical Research Communications 224, 338-334.

Gugliucci, A., Bastos, D.H., Schulze, J., Souza, M.F., 2009. Caffeic and chlorogenic acids in *Ilex paraguariensis* extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. Fitoterapia 80, 339-344.

Gugliucci, A., Bastos, D.H., 2009. Chlorogenic acid protects paraoxonase 1 activity in high density lipoprotein from inactivation caused by physiological concentrations of hypochlorite. Fitoterapia 80,138-142.

Gugliucci, A., Menini, T., 2002. The botanical extracts of *Achyrocline satureoides* and *Ilex paraguariensis* prevent methylglyoxal-induced inhibition of plasminogen and antithrombin III. Life Science 72, 279-292.

Gugliucci, A., Stahl, A. J., 1995. Low density lipoprotein oxidation is inhibited by extracts of *Ilex paraguariensis*. Biochemistry and Molecular Biology International 35, 47-56.

Gülçin, I., 2011. Antioxidant activity of food constituents: an overview. Archives in Toxicology 86, 345-391.

Haddy, F.J., Vanhoutte, P.M., Feletou, M., 2006. Role of potassium in regulating blood flow and blood pressure. The American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 290, 546-552.

Halliwell, B., 2014. Cell culture, oxidative stress, and antioxidants: avoiding pitfalls. Biomedical Journal 37, 99-105.

Häkkinen, S.H., Kärenlampi, S.O., Mykkänen, H.M., Törrönen, A.R., 2000. Influence of domestic processing and storage on flavonol contents in berries. Journal of Agricultural and Food Chemistry 48, 2960-2965.

Hansen, A.S., Marckmann, P., Dragsted, L.O., Finne Nielsen, I.L., Nielsen, S.E., Gronbak, M., 2005. Effect of red wine and red grape extract on blood lipids, haemostatic factors, and other risk factors for cardiovascular disease. European Journal of Clinical Nutrition 59, 449-455.

Harborne, J.B., Baxter, H., 1999. Handbook of Natural Flavonoids. 2 vols. Wiley, Chichester. Editor: Harborne, J.B.

Hartojo, W., Silvers, A.L., Thomas, D.G., Seder, C.W., Lin, L., Rao, H., Rehemtulla, A., 2010. Curcumin promotes apoptosis, increases chemosensivity, and inhibits nuclear factor κB in esophageal adenocarcinoma. Translational Oncology 3, 99-108.

Hatzidimitriou E, Nenadis N, Tsimidou MZ. 2007. Changes in the catechin and epicatechin content of grape seeds on storage under different water activity (aw) conditions. Food Chemistry 105, 1504-1511.

Hayek, T., Fuhrman, B., Vaya, J., Rosenblat, M., Belinky, P., Coleman, R., Elis, A., Aviram, M., 1997. Reduced progression of atherosclerosis in apolipoprotein E–deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arteriosclerosis, Thrombosis, and Vascular Biology 17, 2744-2752.

Heck, C.I., González de Mejia, E., 2007. Yerba mate tea (*Ilex paraguariensis*): a comprehensive review on chemistry, health implications, and technological considerations. Journal of Food Science 72, 138-151.

Heckman, M.A., Weil, J., de Mejia, E.G., 2010. Caffeine (1,3,7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. Journal of Food Science 75, 77-87.

Hernandez-Jimenez, A., Gomez-Plaza, E., Martinez-Cutillas, A., Kennedy, J.A., 2009. Grape skin and seed proanthocyanidins from Monastrell x Syrah grapes. Journal of Agricultural and Food Chemistry 57, 10798-10803.

Hertog, M.G., Feskens, E.J., Kromhout, D., 1997. Antioxidant flavonols and coronary heart disease risk. The Lancet 349, 699.

Hirrlinger, J., Resch, A., Gutterer, J.M., Dringen, R., 2002. Oligodendroglial cells in culture effectively dispose of exogenous hydrogen peroxide: comparison with cultured neurones, astroglial and microglial cells. Journal of Neurochemistry 82, 635-644.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214-226.

Hogan, S., Canning, C., Sun, S., Sun, X., Zhou, K., 2010. Effects of grape pomace antioxidant extract on oxidative stress and inflammation in diet induced obese mice. Journal of Agriculture and Food Chemistry 58, 11250–11256.

Holst, B., Williamson, G., 2008. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Current Opinion in Biotechnology 19, 73-82.

Holt, E. M., Steffen, L. M., Moran, A., Basu, S., Steinberger, J., Ross, J. A., Hong, C. P., Sinaiko, A. R., 2009. Fruit and vegetable consumption and its relation to markers of inflammation and oxidative stress in adolescents. Journal of the American Dietetic Association 109, 414-421.

Hou, J., Li, Y., Zhou, Z., Valiaeva, N., Beadle, J. R., Hostetler, K., Freeman, W. R., Hu, D.-N., Chen, H., Cheng, L., 2011. Antiproliferative property of hexadecyloxypropyl 9-[2-(phosphonomethoxy) ethyl] guanine (HDP-PMEG) for unwanted ocular proliferation. Molecular Vision 17, 627-637.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A., Prior, R.L., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. Journal of Agricultural and Food Chemistry 50, 4437-4444.

Huang, D., Ou, B., Prior, R. L., 2005. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry 53, 1841-1856.

Huang, J., de Paulis, T., May, J.M., 2004. Antioxidant effects of dihydrocaffeic acid in human EA.hy926 endothelial cells. Journal of Nutritional Biochemistry 15, 722-729.

Huang, P.L., 2003. Endothelial nitric oxide synthase and endothelial dysfunction. Current Hypertension Reports 5, 473-480.

Huisman, A., Van De Wiel, A., Rabelink, T.J., Van Faassen, E.E., 2004. Wine polyphenols and ethanol do not significantly scavenge superoxide nor affect endothelial nitric oxide production. The Journal of Nutritional Biochemistry 15, 426-432.

Inoue, M., Noda, M., Kurahashi, N., Iwasaki, M., Sasazuki, S., Iso, H., Tsugane, S., Japan Public Health Center-Based Prospective Study Group, 2009. Impact of metabolic factors on subsequent cancer risk: results from a large-scale population-based cohort study in Japan. European Journal of Cancer Prevention 18, 240-247.

Inoue, M., Suzuki, R., Sakaguchi, N., Li, Z., Takeda, T., Ogihara, Y., Jiang, B.Y., Chen, Y., 1995. Selective induction of cell death in cancer cells by gallic acid. Biological & Pharmaceutical Bulletin 18, 1526-1530.

Isogawa, A., Noda, M., Takahashi, Y., Kadowaki, T., Tsugane, S., 2003. Coffee consumption and risk of type 2 diabetes mellitus. The Lancet 361, 703-704.

Isuzugawa, K., Inoue, M., Ogihara, Y., 2001. Catalase contents in cells determine sensitivity to the apoptosis inducer gallic acid. Biological & Pharmaceutical Bulletin 24, 1022-1026.

Ito, E., Crozier, A., Ashihara, H., 1997. Theophylline metabolism in higher plants. Biochimica et Biophysica Acta 1336, 323-330.

Itoh, A., Isoda, K., Kondoh, M., Kawase, M., Watari, A., Kobayashi, M., Tamesada, M., Yagi, K., 2010. Hepatoprotective effect of syringic acid and vanillic acid on CCl4-induced liver injury. Biological & Pharmaceutical Bulletin 33, 983-987.

Ivanova, V., Stefova, M., Vojnoski, B., Dörnyei, Á., Márk, L., Dimovska, V., Stafilov, T., Kilár, F., 2011. Identification of polyphenolic compounds in red and white grape varieties grown in R. Macedonia and changes of their content during ripening. Food Research International 44, 2851-2860.

Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., Fujita, T., 2004. In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. Journal of Agricultural and Food Chemistry 52, 4893-4898.

Jackson, D., Lombard, P., 1993. Environmental and management practices affecting grape composition and wine quality-a review. American Journal of Enology and Viticulture 44, 409-430.

Jia, Z., Nallasamy, P., Liu, D., Shah, H., Li, J. Z., Chitrakar, R., Si, H., McCormick, J., Zhu, H., Zhen, W., Li, Y., 2015. Luteolin protects against vascular inflammation in mice and TNF-alpha-induced monocyte adhesion to endothelial cells via suppressing IKappaBalpha/NF-kappaB signaling pathway. The Journal of Nutritional Biochemistry 26, 293-302.

Jin, C.F., Shen, S.R., Sr., Zhao, B.L., 2001. Different effects of five catechins on 6-hydroxydopamine-induced apoptosis in PC12 cells. Journal of Agricultural and Food Chemistry 49, 6033-6038.

Joe, A.K., Liu, H., Suzui, M., Vural, M.E., Xiao, D., Weinstein, I.B., 2002. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. American Association for Cancer Research 8, 893-903.

Johnston, K.L., Clifford, M.N., Morgan, L.M., 2003. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. The American Journal of Clinical Nutrition 78, 728-733.

Johnston, T.V., Morris, J.R., 1997. HPLC Analysis of Cabernet Sauvignon and Noble Wine pigment fractions. Journal of Food Science 62, 684-687.

Kamangar, F., Schantz, M.M., Abnet, C.C., Fagundes, R.B., Dawsey, S.M., 2008. High levels of carcinogenic polycyclic aromatic hydrocarbons in mate drinks. Cancer Epidemiology, Biomarkers & Prevention 17, 1262-1268

Kammerer, D., Claus, A., Carle, R., Schieber, A., 2004. Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera L*.) by HPLC-DAD-MS/MS. Journal of Agricultural and Food Chemistry 52, 4360-4367.

Kampa, M., Nifli, A.P., Notas, G., Castanas, E., 2007. Polyphenols and cancer cell growth. Reviews of Physiology, Biochemistry, Pharmacology 159, 79-113.

Kane, M. O., Anselm, E., Rattmann, Y. D., Auger, C., Schini-Kerth, V. B., 2009. Role of gender and estrogen receptors in the rat aorta endothelium-dependent relaxation to red wine polyphenols. Vascular pharmacology 51, 140-146.

Kang, Y.R., Lee, H.Y., Kim, J.H., Moon, D.I., Seo, M.Y., Park, S.H., Choi, K.H., Kim, C.R., Kim, S.H., Oh, J.H., Cho, S.W., Kim, S.Y., Kim, M.G., Chae, S.W., Kim, O., Oh, H.G., 2012. Anti-obesity and anti-diabetic effects of Yerba Mate (*Ilex paraguariensis*) in C57BL/6J mice fed a high-fat diet. Laboratory Animal Research 28, 23-29.

Karbach, S., Jansen, T., Horke, S., Heeren, T., Scholz, A., Coldewey, M., Karpi, A., Hausding, M., Kroller-Schon, S., Oelze, M., Munzel, T., Daiber, A., 2012. Hyperglycemia and oxidative stress in cultured endothelial cells - a comparison of primary endothelial cells with an immortalized endothelial cell line. Journal of Diabetes and Its Complications 26, 155-162.

Kar, P., Laight, D., Rooprai, H.K., Shaw, K.M., Cummings, M., 2009. Effects of grape seed extract in type 2 diabetic subjects at high cardiovascular risk: a double blind randomized placebo controlled trial examining metabolic markers, vascular tone, inflammation, oxidative stress and insulin sensitivity. Diabetic Medicine 26, 526-531.

Karthikeyan, K., Sarala Bai, B.R., Devaraj, S.N., 2009. Cardioprotective effect of grape seed procyanidins on isoproernol-induced myocardial injury in rats. Journal of Cardiovascular Pharmacology 53, 109–115.

Kawser, H. M., Abdal, D.A., Han, J., Yin, Y., Kim, K., Kumar S. S., Yang, G.M., Choi, H.Y., Cho, S.G., 2016. Molecular mechanisms of the anti-obesity and anti-diabetic properties of flavonoids. International Journal of Molecular Sciences 17, 569-600.

Kempf, K., Herder, C., Erlund, I., Kolb, H., Martin, S., Carstensen, M., Koenig, W., Sundvall, J., Bidel, S., Kuha, S., Tuomilehto, J., 2010. Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. The American Journal of Clinical Nutrition 91, 950-957.

Kennedy, J.A., Hayasaka, Y., Vidal, S., Waters, E.J., Jones, G.P., 2001. Composition of grape skin proanthocyanidins at different stages of berry development. Journal of Agricultural and Food Chemistry 49, 5348-5355.

Khan, H.Y., Zubair, H., Faisal, M., Ullah, M.F., Farhan, M., Sarkar, F.H., Ahmad, A., Hadi, S.M., 2014. Plant polyphenol induced cell death in human cancer cells involves mobilization of intracellular copper ions and reactive oxygen species generation: a mechanism for cancer chemopreventive action. Molecular Nutrition & Food Research 58, 437-446.

Klimczak, I., Małecka, M., Szlachta, M., Gliszczyńska-Świgło, A., 2007. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. Journal of Food Composition and Analysis 20, 313-322.

Knasmuller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., Mersch-Sundermann, V., Williamson, G., Hietsch, G., Langer, T., Darroudi, F., Natarajan, A.T., 1998. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutation Research 402, 185-202.

Kokkonen, H., Soderstrom, I., Rocklov, J., Hallmans, G., Lejon, K., Rantapaa Dahlqvist, S., 2010. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. Arthritis and Rheumatism 62, 383-391.

Konishi, Y., Kobayashi, S., 2004. Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal Caco-2 cell monolayers. Journal of Agricultural and Food Chemistry 52, 2518-2526.

Kovacs, E.M., Lejeune, M.P., Nijs, I. Westerterp-Plantenga, M.S., 2004. Effects of green tea on weight maintenance after body-weight loss. British Journal of Nutrition 91, 431-437.

Kozuma, K., Tsuchiya, S., Kohori, J., Hase, T., Tokimitsu, I., 2005. Antihypertensive effect of green coffee bean extract on mildly hypertensive subjects. Hypertension Research 28, 711-718.

Krishnamurthy, P., Wadhwani, A., 2012. Antioxidant enzymes and human health. In: *Biochemistry, Genetics and Molecular Biology – Antioxidant Enzyme.* M. A. El Missiry (Ed.) INTECH Open Access Publisher, chapter 1, 1-16.

Krstic, M., Stojadinovic, M., Smiljanic, K., Stanic-Vucinic, D., Cirkovic Velickovic, T., 2015. The anti-cancer activity of green tea, coffee and cocoa extracts on human cervical adenocarcinoma HeLa cells depends on both pro-oxidant and anti-proliferative activities of polyphenols. Royal Society of Chemistry Advances 5, 3260-3268.

Kuhnau, J., 1976. The flavonoids: a class of semi-essential food components: their role in human nutrition. World Review Nutriton Diet 24,117-191.

Kuo, P.L., Hsu, Y.L., 2008. The grape and wine constituent piceatannol inhibits proliferation of human bladder cancer cells via blocking cell cycle progression and inducing Fas/membrane bound Fas ligand-mediated apoptotic pathway. Molecular Nutrition & Food Research 52, 408-418.

Kurahashi, N., Inoue, M., Iwasaki, M., Sasazuki, S., Tsugane, S., 2009. Coffee, green tea, and caffeine consumption and subsequent risk of bladder cancer in relation to smoking status: a prospective study in Japan. Cancer Science 100, 294-291.

Kurt, A., Speer, K., 2002. Untersuchungen zum einfluss der dampfungsparameter auf die diterpengehalte von Arabica Roh- und Rostkaffees. Dtsch. Lebensmittel-Rdsch. 98, 1-4.

Ky, I., Teissedre, P.L., 2015. Characterisation of Mediterranean grape pomace seed and skin extracts: polyphenolic content and antioxidant activity. Molecules 20, 2190-2207.

Ladurner, A., Schachner, D., Schueller, K., Pignitter, M., Heiss, E.H., Somoza, V., Dirsch, V.M., 2014. Impact of trans-resveratrol-sulfates and -glucuronides on endothelial nitric oxide synthase activity, nitric oxide release and intracellular reactive oxygen species. Molecules 19, 16724-16736.

Lakshmi, B.V.S., Sudhakar, M., Anisha, M., 2014. Neuroprotective role of hydroalcoholic extract of *Vitis vinifera* against aluminium-induced oxidative stress in rat brain. Neuro Toxicology 41, 73-79.

Lampe, J. W., Chang, J.-L., 2007. Interindividual differences in phytochemical metabolism and disposition. Seminars in Cancer Biology 17, 347-353.

Lang, R., Dieminger, N., Beusxh, A., Lee, Y.-M., Dunkel, A., Suess, B., Skurk, T., Wahl, A., Hauner, H., Hofmann, T., 2013. Bioappearance and pharmacokinetics of bioactives upon coffee consumption. Analytical and Bioanalytical Chemistry 405, 8487-8503.

Larsson, S.C., Andersson, S.O., Johansson, J.E., Wolk, A., 2008. Fruit and vegetable consumption and risk of bladder cancer: a prospective cohort study. Cancer Epidemiology, Biomarkers & Prevention 17, 2519-2522.

Larsson, S.C., Hakansson, N., Naslund, I., Bergkvist, L., Wolk, A., 2006. Fruit and vegetable consumption in relation to pancreatic cancer risk: a prospective study. Cancer Epidemiology, Biomarkers & Prevention 15, 301-305.

Lee, K.J., Inoue, M., Otani, T., Iwasaki, M., Sasazuki, S., Tsugane, S., 2007. Coffee consumption and risk of colorectal cancer in a population-based prospective cohort of Japanese men and women. International Journal of Cancer 121, 1312-1318.

Leikert, F., J., 2002. Red Wine Polyphenols Enhance Endothelial Nitric Oxide Synthase Expression and Subsequent Nitric Oxide Release From Endothelial Cells. Circulation 106, 1614-1617.

Leitao, A.C., Braga, R.S., 1994. Mutagenic and genotoxic effects of mate (*Ilex paraguariensis*) in prokaryotic organisms. Brazilian Journal of Medical and Biological Research 27, 1517-1525.

León, K., Mery, D., Pedreschi, F., León, J., 2006. Color measurement in L∗a∗b∗ units from RGB digital images. Food Research International 39, 1084-1091.

León-González, A.J., Mateos, R., Ramos, S., Martín, M.A., Sarriá, B., Martín-Cordero, C., López-Lázaro, M., Bravo, L., Goya, L., 2012. Chemo-protective activity and characterization of phenolic extracts from *Corema album.* Food Research International 49, 728-738.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology 186, 464-478.

Lewandowska, U., Szewczyk, K., Hrabec, E., Janecka, A., Gorlach, S., 2013. Overview of metabolism and bioavailability enhancement of polyphenols. Journal of Agricultural and Food Chemistry 61, 12183-12199.

Li, F., Li, S., Li, H. B., Deng, G. F., Ling, W. H., Xu, X. R., 2013. Antiproliferative activites of tea and herbal infusions. Food & Function 4, 530-538.

Liang, Z., Cheng, L., Zhong, G.Y., Liu, R.H., 2014. Antioxidant and antiproliferative activities of twenty-four *Vitis vinifera* grapes. PLoS One 9, 105-146.

Li, G., Zhu, Y., Zhang, Y., Lang, J., Chen, Y., Ling, W., 2013. Estimated daily flavonoid and stilbene intake from fruits, vegetables, and nuts and associations with lipid profiles in Chinese adults. Journal of the Academy of Nutrition and Dietetics 113, 786-794.

Li, H., Xia, N., Brausch, I., Yao, Y., Forstermann, U., 2004. Flavonoids from artichoke (*Cynara scolymus* L.) up-regulate endothelial-type nitric-oxide synthase gene expression in human endothelial cells. Journal of Pharmacology and Experimental Therapeutics 310, 926-932.

Li, S.I., Chang, C.Q., Fu-Ying, M.A., Chang-Long, Y U., 2009. Modulating effects of chlorogenic acid on lipids and glucose metabolism and expression of hepatic peroxisome proliferator-activated receptor-α in golden hamsters fed on high fat diet. Biomedical and Environmental Sciences 22, 122-129.

Lima, C.F., Fernandes-Ferreira, M., Pereira-Wilson, C., 2006. Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels. Life Sciences 79, 2056-2068.

Loria, D., Barrios, E., Zanetti, R., 2009. Cancer and yerba mate consumption: a review of possible associations. Revista Panamericana de Salud Publica 25, 530-539.

Lu, Z., Nie, G., Belton, P. S., Tang, H., & Zhao, B., 2006. Structure-activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochemistry International 48, 263-274.

Luo, J., Inoue, M., Iwasaki, M., Sasazuki, S., Otani, T., Ye, W., Tsugane, S., 2007. Green tea and coffee intake and risk of pancreatic cancer in a large-scale, population-based cohort study in Japan (JPHC study). European Journal of Cancer Prevention 16, 542-548.

Makris, D.P., Boskou, G., Andrikopoulos, N.K., 2007. Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. Journal of Food Composition and Analysis 20, 125-132.

Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C., Remesy, C., 2003. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. European Journal of Clinical Nutrition 57, 235-242.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. The American Journal of Clinical Nutrition 79, 727-747.

Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The American Journal of Clinical Nutrition 81, 230s-242s.

Mariappan, N., Soorappan, R.N., Haque, M., Sriramula, S., Francis, J., 2007. TNF-alpha-induced mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase mimetic Tempol. American Journal of Physiology-Heart and Circulatory Physiology 293, 2726-2737.

Martin, M.A., Fernandez-Millan, E., Ramos, S., Bravo, L., Goya, L., 2014. Cocoa flavonoid epicatechin protects pancreatic beta cell viability and function against oxidative stress. Molecular Nutrition & Food Research 58, 447-456.

Martin, M.A., Serrano, A.B., Ramos, S., Izquierdo-Pulido, M., Bravo, L., Goya, L., 2010. Cocoa flavonoids up-regulate antioxidant enzymes activity via ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. Journal of Nutritional Biochemistry. 21, 196-205.

Martin, M.A., Goya, L., Ramos, S., 2016. Anti-diabetic actions of cocoa flavanols. Molecular Nutrition & Food Research 60,1756-1769.

Martin, M.A., Ramos, S., Cordero-Herrero, I., Bravo, L., Goya, L., 2013. Cocoa phenolic extract protects pancreatic beta cells against oxidative stress. Nutrients 5, 2955-2968.

Martin, M.A., Ramos, S., Mateos, R., Izquierdo-Pulido, M., Bravo, L., Goya, L., 2010a. Protection of human HepG2 cells against oxidative stress by the flavonoid epicatechin. Phytotherapy Research 24, 503-509.

Martin, M.A., Ramos, S., Rodríguez-Ramiro, I., Mateos, R., Bravo, L., Goya, L., 2012. Signal transduction pathways involved in the chemo-preventive effect of dietary antioxidants: study in HepG2 as a cell culture model. Current Nutrition & Food Science 8, 112-121.

Martin, M.A., Serrano, A.B., Ramos, S., Pulido, M.I., Bravo, L., Goya, L., 2010b. Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. Journal of Nutritional Biochemistry 21, 196-205.

Martinet, A., Hostettmann, K., Schutz, Y., 1999. Thermogenic effects of commercially available plant preparations aimed at treating human obesity. Phytomedicine 6, 231-238.

Martínez-López, S., 2014. Cacao y café, alimentos ricos en fitoquímicos con propiedades beneficiosas en salud. Estudios de biodisponibilidad de metilxantinas y efectos en salud en voluntarios sanos y con riesgo cardiovascular. Doctoral Thesis, Universidad Complutense de Madrid.

Martinez-Lopez, S., Sarria, B., Baeza, G., Mateos, R., Bravo-Clemente, L., 2014. Pharmacokinetics of caffeine and its metabolites in plasma and urine after consuming a soluble green/roasted coffee blend by healthy subjects. Food Research International 64, 125-133.

Martínez-López, S., Sarriá, B., Mateos, R., Bravo, L. Sustained consumption of a soluble green/roasted coffee blend reduces cardiovascular risk in healthy and hypercholesterolemic subjects. Unpublished results.

Martins, F., Suzan, A.J., Cerutti, S.M., Arçari, D.P., Ribeiro, M.L., Bastos, D.H., Carvalho, P.O., 2009. Consumption of mate tea (*Ilex paraguariensis*) decreases the oxidation of unsaturated fatty acids in mouse liver. British Journal of Nutrition 101, 527-532.

Masella, R., Di Benedetto, R., Varì, R., Filesi, C., Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. Journal of Nutritional Biochemistry 16, 577-586.

Mateos, R., Goya, L., Bravo, L., 2006. Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic and ferulic acids) by HepG2 cells as a model of the human liver. Journal of Agricultural and Food Chemistry 54, 8724-8732.

Mateos, R., Pereira-Caro, G., Bacon, J.R., Bongaerts, R., Sarriá, B., Bravo, L., Kroon, P.A. Anticancer activity of olive oil hydroxytyrosyl acetate in human adenocarcinoma Caco-2 cells. Journal of Agricultural Food Chemistry 61, 3264-3269.

Mateos, R., Pereira-Caro, G., Saha, S., Cert, R., Redondo-Horcajo, M., Bravo, L., Kroon, P.A., 2011. Acetylation of hydroxytyrosol enhances its transport across differentiated Caco-2 cell monolayers. Food Chemistry 125, 865-872.

Mathew, R., Karantza-Wadsworth, V., White, E., 2007. Role of authophagy in cancer. Nature Reviews Cancer 7, 961-967.

Matsumoto, R.L., Bastos, D.H., Mendonça, S., Nunes, V.S., Bartchewsky, W., Ribeiro, M.L., Carvalho P.O., 2009. Effects of mate tea (*Ilex paraguariensis*) ingestion on mRNA expression of antioxidant enzymes, lipid peroxidation, and total antioxidant status in healthy young women. Journal of Agricultural and Food Chemistry 57, 1775-1780.

Matsumoto, R.L., Mendonca, S., de Oliveira, D.M., Souza, M.F., Bastos, D.H., 2009. Effects of mate tea intake on *ex vivo* LDL peroxidation induced by three different pathways. Nutrients 1, 18-29.

Mazzafera, P., 1997. Mate drinking: caffeine and phenolic acid intake. Food Chemistry 60, 67–71.

McDougall, G.J., Dobson, P., Smith, P., Blake, A., Stewart, D., 2005. Assessing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. Journal of Agricultural and Food Chemistry 53, 5896-5904.

Melo, P.S., Massarioli, A.P., Denny, C., dos Santos, L.F., Franchin, M., Pereira, G.E., Vieira, T.M., Rosalen, P.L., de Alencar, S.M., 2015. Winery by-products: extraction optimization, phenolic composition and cytotoxic evaluation to act as a new source of scavenging of reactive oxygen species. Food Chemistry 181,160-169.

Meydani, M., Hasan, S.T., 2010. Dietary polyphenols and obesity. Nutrients 2, 737-751.

Miccadei, S., Di Venere, D., Cardinali, A., Romano, F., Durazzo, A., Foddai, M.S., Fraioli, R., Mobarhan, S., Maiani, G., 2008. Antioxidative and apoptotic properties of polyphenolic extracts from edible part of artichoke (*Cynara scolymus* L.) on cultured rat hepatocytes and on human hepatoma cells. Nutrition and Cancer 60, 276-283.

Mildner-Szkudlarz, S., Zawirska-Wojtasiak, R., Szwengiel, A., Pacyński, M., 2011. Use of grape by-product as a source of dietary fibre and phenolic compounds in sourdough mixed rye bread. International Journal of Food Science & Technology 46, 1485-1493.

Mineharu, Y., Koizumi, A., Wada, Y., Iso, H., Watanabe, Y., Date, C., Yamamoto, A., Kikuchi, S., Inaba, Y., Toyoshima, H., Kondo, T., Tamakoshi, A., 2009. Coffee, green tea, black tea and oolong tea consumption and risk of mortality from cardiovascular disease in Japanese men and women. Journal of Epidemiology & Community Health 65, 230-40.

Miranda, D.D., Arçari, D.P., Pedrazzoli, J.Jr., Carvalho, P.O., Cerutti, S.M., Bastos, D.H., Ribeiro M.L., 2008. Protective effects of mate tea (*Ilex paraguariensis*) on H_2O_2-induced DNA damage and DNA repair in mice. Mutagenesis 23, 261-265.

Monagas, M., Hernández-Ledesma, B., Gómez-Cordovés, C., Bartolomé, B., 2005. Commercial dietary ingredients from *Vitis vinifera* L. leaves and grape skins: antioxidant and chemical characterization. Journal of Agricultural and Food Chemistry 54, 319-327.

Monteiro, M., Farah, A., Perrone, D., Trugo, L.C., Donangelo, C., 2007. Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. The Journal of Nutrition 137, 2196-2201.

Moore, J., Megaly, M., MacNeil, A.J., Klentrou, P., Tsiani, E., 2016. Rosemary extract reduces Akt/mTOR/p70S6K activation and inhibits proliferation and survival of A549 human lung cancer cells. Biomedicine & Pharmacotherapy 83, 725-732.

Morais, H., Ramos, C., Forgacs, E., Cserhati, T., Oliviera, J., 2002. Influence of storage conditions on the stability of monomeric anthocyanins studied by reversed-phase high-performance liquid chromatography. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 770, 297-301

Mosimann, A.L., Wilhelm-Filho, D., da Silva, E.L., 2006. Aqueous extract of *Ilex paraguariensis* attenuates the progression of atherosclerosis in cholesterol-fed rabbits. Biofactors 26, 59-70.

Muccillo-Baisch, A.L., Johnston, K.B., Paganini-Stein F.L., 1998. Endothelium-dependent vasorelaxing activity of aqueous extracts of *Ilex paraguariensis* on mesenteric arterial bed of rats. Journal of Ethnopharmacology 60, 133–139.

Murad, L.D., Soares, N.C.P., Brand, C., Monteiro, M.C., Teodoro, A.J., 2015. Effects of caffeic and 5-caffeoylquinic acids on cell viability and cellular uptake in human colon adenocarcinoma cells. Nutrition Cancer 67, 532-542.

Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Moriarty, H., Pike, M. J., Turner, A. H., Mann, N. J., Sinclair, A. J., 2003. Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. The American Journal of Clinical Nutrition 77, 1466-1473.

Nakachi, K., Matsuyama, S., Miyake, S., Suganuma, M., Imai, K., 2000. Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention. Biofactors 13, 49-54.

Nakatsu, N., Nakamura, T., Yamazaki, K., Sadahiro, S., Makuuchi, H., Kanno, J., Yamori, T., 2007. Evaluation of action mechanisms of toxic chemicals using JFCR39, a panel of human cancer cell lines. Molecular Pharmacology 72, 1171-1180.

Nardini, M., Scaccini, C., Virgili, F., 2002. Modulation of kinase activity by polyphenols. Annals of the New York Academy of Sciences 973, 368-370.

Narita, Y., Inouye, K., 2009. Kinetic analysis and mechanism on the inhibition of chlorogenic acid and its components against porcine pancreas α-amylase isozymes I and II. Journal of Agricultural and Food Chemistry 57, 9218-9225.

Naseem, K.M., 2005. The role of nitric oxide in cardiovascular diseases. Molecular Aspects of Medicine 26, 33-65.

Negro, L.T., Micel, A., 2003. Phenolic compounds and antioxidant activity from red grape marc extracts. Bioresource Technology 87, 41-44.

Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., Scalbert, A., 2010. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database : the Journal of Biological Databases and Curation 2010, bap024.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L., Exon, J.H., 2006. A review of the effects and mechanisms of polyphenolics in cancer. Critical Reviews in Food Science and Nutrition 46, 161-183.

Nkondjock, A., 2009. Coffee consumption and the risk of cancer: an overview. Cancer Letter 277, 121-125.

Obreque-Slier, E.A., Peña-Neira, A.I., López-Solís, R., Zamora-Marín, F., Ricardo-da Silva, J.M., Laureano, O., 2010. Comparative study of the phenolic composition of seeds and skins from carménère and cabernet sauvignon grape varieties (*Vitis vinifera* L.) during ripening. Journal Agricultural of Food Chemistry 58, 3591-3599.

Ochiai, R., Chikama, A., Kataoka, K., Tokimitsu, I., Maekawa, Y., Ohishi, M., Rakugi, H., Mikami, H., 2009. Effects of hydroxyhydroquinone-reduced coffee on vasoreactivity and blood pressure. Hypertension Research 32, 969-974.

Ochiai, R., Jokura, H., Suzuki, A., Tokimitsu, I., Ohishi, M., Komai, N., Rakugi, H., Ogihara, T., 2004. Green coffee bean extract improves human vasoreactivity. Hypertension Research 27, 731-737.

Oliveira, D.M., Freitas, H.S., Souza, M.F.F., Arçari, D.P., Ribeiro, M.L., Carvalho, P.O., Bastos, D.H.M., 2008a. Yerba maté (*Ilex paraguariensis*) aqueous extract

decreases intestinal SGLT1 gene expression but does not affect other biochemical parameters in alloxan-diabetic wistar rats. Journal of Agricultural and Food Chemistry 56, 10527-10533.

Oliveira, I., Sousa, A., Ferreira, I.C., Bento, A., Estevinho, L., Pereira, J.A., 2008b. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. Food and Chemical Toxicology, 46, 2326-2331.

Olthof, M. R., Hollman, P. C., Katan, M. B., 2001. Chlorogenic acid and caffeic acid are absorbed in humans. The Journal of Nutrition 131, 66-71.

Paganini-Stein, F.L., Schmidt, B., Furlong, E.B., Souza-Soares, L.A., Soares, M.C., Vaz, M.R., Muccillo-Baisch, A.L., 2005. Vascular responses to extractable fractions of *Ilex paraguariensis* in rats fed standard and high-cholesterol diets. Biological Research For Nursing 7, 146-56.

Pai, J.K., Hankinson, S.E., Thadhani, R., Rifai, N., Pischon, T., Rimm, E.B., 2006. Moderate alcohol consumption and lower levels of inflammatory markers in US men and women. Atherosclerosis 186, 113-120.

Palthur, M. P., Palthur, S. S., Chitta, S. K., 2010. Nutraceuticals: a conceptual definition. International Journal of Pharmacy and Pharmaceutical Sciences 2, 19-27.

Pandey, K.B., Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity 2, 270-278.

Paneni, F., Costantino, S., Cosentino, F., 2014. Molecular mechanisms of vascular dysfunction and cardiovascular biomarkers in type 2 diabetes. Cardiovascular Diagnosis and Therapy 4, 324-332.

Panicker, S.R., Sreenivas, P., Babu, M.S., Karunagaran, D., Kartha, C.C., 2010. Quercetin attenuates Monocyte Chemoattractant Protein-1 gene expression in glucose primed aortic endothelial cells through NF-kappaB and AP-1. Pharmacological Research 62, 328-336.

Park, J.B., 2009. 5-Caffeoylquinic acid and caffeic acid orally administered suppress P-selectin expression on mouse platelets. Journal of Nutritional Biochemistry 20, 800-805.

Park, J. B., 2013. Isolation and quantification of major chlorogenic acids in three major instant coffee brands and their potential effects on H_2O_2-induced mitochondrial membrane depolarization and apoptosis in PC-12 cells. Food & function 4, 1632-1638.

Park, C., Kim, G.Y., Kim, G.D., Choi, B.T., Park, Y.M., Choi, Y.H., 2006. Induction of G2/M arrest and inhibition of cyclooxygenase-2 activity by curcumin in human bladder cancer T24 cells. Oncology Reports 15, 1225-1231.

Passamonti, S., Vrhovsek, U., Vanzo, A., Mattivi, F., 2003. The stomach as a site for anthocyanins absorption from food. FEBS Letter 544, 210-213.

Paur, I., Balstad, T. R., Kolberg, M., Pedersen, M. K., Austenaa, L. M., Jacobs, D. R., Blomhoff, R., 2010. Extract of Oregano, Coffee, Thyme, Clove, and Walnuts Inhibits NF-κB in Monocytes and in Transgenic Reporter Mice. Cancer Prevention Research 3, 653-663.

Peng, C.C., Chyau, C.C., Wang, H.E., Chang, C.H., Chen, K.C., Chou, K.Y., Peng, R.Y., 2013. Cytotoxicity of ferulic acid on T24 cell line differentiated by different microenvironments. BioMed Research International 2013, art. ID 579859, 1-7.

Pereira-Caro, G., Mateos, R., Saha, S., Madrona, A., Espartero, J.L., Bravo, L., Kroon, P.A., 2010. Transepithelial transport and metabolism of new lipophilic ether derivatives of hydroxytyrosol by enterocyte-like Caco-2/TC7 cells. Journal of Agriculture and Food Chemistry 58, 11501-11509.

Pereira-Caro, G., Mateos, R., Sarria, B., Cert, R., Goya, L., Bravo, L., 2012a. Hydroxytyrosyl acetate contributes to the protective effects against oxidative stress of virgin olive oil. Food Chemistry 131, 869-878.

Pereira-Caro, G., Sarria, B., Madrona, A., Espartero, J.L., Escuderos, M.E., Bravo, L., Mateos, R., 2012b. Digestive stability of hydroxytyrosol, hydroxytyrosyl acetate and alkyl hydroxytyrosyl ethers. International Journal of Food Science and Nutrition 63, 703-707.

Perez-Jimenez, J., Fezeu, L., Touvier, M., Arnault, N., Manach, C., Hercberg, S., Galan, P., Scalbert, A., 2011. Dietary intake of 337 polyphenols in French adults. The American Journal of Clinical Nutrition 93, 1220-1228.

Perez-Vicente, A., Gil-Izquierdo, A., Garcia-Viguera, C., 2002. In vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. Journal of Agricultural and Food Chemistry 50, 2308-2312.

Perez-Vizcaino, F., Duarte, J., Santos-Buelga, C., 2012. The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids. Journal of the Science of Food and Agriculture 92, 1822-1825.

Perrone, D., Donangelo, R., Donangelo, C.M., Farah, A., 2010. Modeling weight loss and chlorogenic acids content in coffee during roasting. Journal of Agricultural and Food Chemistry 58, 12238-12243.

Peters, U., Poole, C., Arab, L., 2001. Does tea affect cardiovascular disease? A meta-analysis. American Journal of Epidemiology 154, 495-503.

Philips, B.J., Coyle, C.H., Morrisroe, S.N., Chancellor, M.B., Yoshimura, N., 2009. Induction of apoptosis in human bladder cancer cells by green tea catechins. Biomedical Research 30, 207-215.

Pignatelli, P., Di Santo, S., Buchetti, B., Sanguigni, V., Brunelli, A., Violi, F., 2006. Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. The FASEB Journal 20, 1082-1089.

Pimentel, G. D., Lira, F. S., Rosa, J. C., Caris, A. V., Pinheiro, F., Ribeiro, E. B., Nascimento, C. M., Oyama, L. M., 2013. Yerba mate extract (*Ilex paraguariensis*) attenuates both central and peripheral inflammatory effects of diet-induced obesity in rats. Journal of Nutritional Biochemistry 24, 809–818.

Pinacho, R., Cavero, R.Y., Astiasarán, I., Ansorena, D., Calvo, M.I., 2015. Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of in vitro digestion on their antioxidant capacity. Journal of Functional Foods 19, 49-62.

Pintos, J., Franco, E.L., Oliveira, B.V., Kowalski, L.P., Curado, M.P., Dewar, R., 1994. Mate, coffee, and tea consumption and risk of cancers of the upper aerodigestive tract in southern Brazil. Epidemiology, 5, 583-590.

Pittler, M.H., Ernst, E., 2004. Dietary supplements for body-weight reduction: a systematic review. The American Journal of Clinical Nutrition 79, 529-536.

Prieur, C., Rigaud, J., Cheynier, V., Moutounet, M., 1994. Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry 36, 781-784.

Puangpraphant, S., Berhow, M.A., de Mejia, E.G., 2011a. Mate (*Ilex paraguariensis* St. Hilaire) saponins induce caspase-3-dependent apoptosis in human colon cancer cells *in vitro*. Food Chemistry 125, 1171-1178.

Puangpraphant, S., Berhow, M.A., Vermillion, K., Potts, G., Gonzalez de Mejia, E., 2011b. Dicaffeoylquinic acids in yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells. Molecular Nutrition & Food Research 55, 1509-1522.

Puangpraphant, S., Berhow, M.A., de Mejia, E., 2012. Yerba mate (*Ilex paraguariensis*) saponins inhibit human colon cancer cell. Hispanic Foods: Chemistry and Bioactive Compounds Chapter 18, 307-321.

Pulido, R., Bravo, L., Saura-Calixto, F., 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry 48, 3396-3402.

Ragione, F.D., Cucciolla, V., Borriello, A., Pietra, V.D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., Zappia, V., 1998. Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochemical and Biophysical Research Communications 250, 53-58.

Rahman, T., Hosen, I., Islam, M.M.T., Shekhar, H.U., 2012. Oxidative stress and human health. Advances in Bioscience and Biotechnology 3, 997-1109.

Ramalakshmi, K., Kubra, I.R., Rao, L.J., 2007. Physicochemical characteristics of green coffee: comparison of graded and defective beans. Journal of Food Science 72, 333-337.

Ramos, S., Alía, M., Bravo, L., Goya, L., 2005. Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). Journal of Agricultural and Food Chemistry 53, 1271-1280.

Ramos, S., 2008. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Molecular Nutrition & Food Research 52, 507-526.

Ramos, S., Rodríguez-Ramiro, I., Martin, M.A., Goya, L., Bravo, L., 2011. Dietary flavanols exert different effects on antioxidant defenses and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. Toxicology in Vitro 25, 1771-1781.

Randhir, R., Lin, Y.T., Shetty, K., 2004. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. Asia Pacifica Journal of Clinical Nutrition 13, 295-307.

Record, I.R., Lane, J.M., 2001. Simulated intestinal digestion of green and black teas. Food Chemistry 73, 481-486.

Reed, J.D., McDowell, R.T.E., van Soest, P.J., Horvath, P.R.J., 1982. Condensed tannins: A factor limiting the use of cassava forage. Journal of the Science of Food and Agriculture 33, 213-220.

Renaud, D., de Lorgeril, M., 1992. Wine, alcohol, platelets and the French paradox for coronary heart disease. The Lancet 339, 1523-1526.

Renouf, M., Guy, P. A., Marmet, C., Fraering, A. L., Longet, K., Moulin, J., Enslen, M., Barron, D., Dionisi, F., Cavin, C., Williamson, G., Steiling, H., 2010. Measurement of caffeic and ferulic acid equivalents in plasma after coffee consumption: small intestine and colon are key sites for coffee metabolism. Molecular Nutrition and Food Research 54, 760-766.

Renouf, M., Marmet, C., Giuffrida, F., Lepage, M., Barron, D., Beaumont, M., Williamson, G., Dionisi, F., 2014. Dose–response plasma appearance of coffee chlorogenic and phenolic acids in adults. Molecular Nutrition & Food Research 58, 301-309.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26, 1231-1237.

Reunanen, A., Heliövaara, M., Aho, K., 2003. Coffee consumption and risk of type 2 diabetes mellitus. The Lancet 361, 702-703.

Ribeiro, L.F., Ribani, R.H., Francisco, T.M., Soares, A.A., Pontarolo, R. Haminiuk, C.W., 2015. Profile of bioactive compounds from grape pomace (*Vitis*

vinifera and *Vitis labrusca*) by spectrophotometric, chromatographic and spectral analyses. Journal ofChromatography B, Biomedical Sciences and Applications 1007, 72-80.

Ricco, R.A., Wagner M.L., Giberti G.C., Gurni A.A., 1995. Antocianos foliares de *Ilex paraguariensis* St. Hil. Acta Farmacéutica Bonaerense 14, 87-90.

Rockenbach, I.I., Gonzaga, L.V., Rizelio, V.M., Gonçalves, A.E.d.S.S., Genovese, M.I., Fett, R., 2011. Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (*Vitis vinifera* and *Vitis labrusca*) pomace from Brazilian winemaking. Food Research International 44, 897-901.

Rockenbach, I.I., Rodrigues, E., Gonzaga, L.V., Caliari, V., Genovese, M.I., Gonçalves ,AEdSS., Fett, R., 2011. Phenolic compounds content and antioxidant activity in pomace from selected red grapes (*Vitis vinifera* L. and *Vitis labrusca* L.) widely produced in Brazil. Food Chemistry 127, 174-179.

Rodríguez de Sotillo, D.V.R., Hadley, M., 2002. Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. Journal of Nutritional Biochemistry 13, 717-726.

Rodríguez-Montealegre, R., Romero Peces, R., Chacón Vozmediano, J.L., Martínez Gascueña, J., García Romero, E., 2006. Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in a warm climate. Journal of Food Composition and Analysis 19, 687-693.

Rodriguez-Ramiro, I., Martin, M.A., Ramos, S., Bravo, L., Goya, L., 2011a. Comparative effects of dietary flavanols on antioxidant defences and their response to oxidant-induced stress on Caco-2 cells. European Journal of Nutrition 50, 313-322.

Rodriguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., Martin, M.A., 2011b. Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. Journal of Nutritional Biochemistry 22, 1186-1194.

Rodriguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., Martin, M.A., 2012. Procyanidin B2 induces Nrf2 translocation and glutathione S-transferase P1 expression via ERKs and p38-MAPK pathways and protect human colonic cells against oxidative stress. European Journal of Nutrition 51, 881-892.

Rodriguez-Ramiro, I., Ramos, S., Lopez-Oliva, E., Agis-Torres, A., Bravo, L., Goya, L., Martin, M.A., 2013. Cocoa polyphenols prevent inflammation in the colon of azoxymethane-treated rats and in TNF-alpha-stimulated Caco-2 cells. The British Journal of Nutrition 110, 206-215.

Rodríguez-Ramiro, I., Ramos, S., Lopez-Oliva, E., Agis-Torres, A., Gómez-Juaristi, M., Mateos, R., Bravo, L., Goya, L., Martín, M.A., 2011c. Cocoa-rich diet prevents azoxymethane-induced colonic preneoplastic lesions in rats by restraining oxidative stress and cell proliferation and inducing apoptosis. Molecular Nutrition & Food Research 55, 1895-1899.

Rodriguez-Rodriguez, R., Justo, M.L., Claro, C.M., Vila, E., Parrado, J., Herrera, M.D., Alvarez de Sotomayor, M., 2012. Endothelium-dependent vasodilator and antioxidant properties of a novel enzymatic extract of grape pomace from wine industrial waste. Food Chemistry 135, 1044-1051.

Rodriguez-Saona, L.E., Guisti, M.M., Wrolstad, R.E., 1999. Color and Pigment stability of red radish and red fleshed potato anthocyanins in juice model systems. Journal of Food Science 64, 451-456.

Rothwell, J.A., Urpi-Sarda, M., Boto-Ordonez, M., Knox, C., Llorach, R., Eisner, R., Cruz, J., Neveu, V., Wishart, D., Manach, C., Andres-Lacueva, C., Scalbert, A., 2012. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. Database: The Journal of Biological Databases and Curation 2012, bas031.

Ruddarraju, R.R., Murugulla, A.C., Kotla, R., Chandra Babu Tirumalasetty, M., Wudayagiri, R., Donthabakthuni, S., Maroju, R., Baburao, K., Parasa, L.S., 2016. Design, synthesis, anticancer, antimicrobial activities and molecular docking studies of theophylline containing acetylenes and theophylline containing 1,2,3-triazoles with variant nucleoside derivatives. European Journal of Medicinal Chemistry 123, 379-396.

Ruhl, C.E., Everhart, J.E., 2005. Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. Gastroenterology 129, 1928-1936.

Sandhu, A.K., Gu, L., 2010. Antioxidant capacity, phenolic content, and profiling of phenolic compounds in the seeds, skin, and pulp of *Vitis rotundifolia* (Muscadine Grapes) as determined by HPLC-DAD-ESI-MSn. Journal of Agricultural and Food Chemistry 58, 4681-4692.

Sangiovanni, E., Di Lorenzo, C., Colombo, E., Colombo, F., Fumagalli, M., Frigerio, G., Restani, P., Dell'Agli, M., 2015. The effect of in vitro gastrointestinal digestion on the anti-inflammatory activity of *Vitis vinifera* L. leaves. Food & Function 6, 2453-2463.

Sarriá, B., Martínez-López, S., Mateos, R., Bravo, L. Long-term consumption of a green/roasted coffee blend positively affects glucose metabolism and insulin resistance in humans. Food Research International, in press (DOI: 10.1016/j.foodres.2015.12.032)

Sarriá, B., Martínez-López, S., Sierra-Cinos, J.L., Garcia-Diz, L., Goya, L., Mateos, R., Bravo, L., 2015. Effects of bioactive constituents in functional cocoa products on cardiovascular health in humans. Food Chemistry 174, 214-218.

Sarriá, B., Martínez-López, S., Sierra-Cinos, J.L., García-Diz, L., Mateos, R., Bravo, L. Regularly consuming a green/roasted coffee blend shows benefits against metabolic syndrome. European Journal of Nutrition, submitted.

Sarria, B., Mateos, R., Gallardo, E., Ramos, S., Martin, M. A., Bravo, L., Goya, L., 2012. Nitroderivatives of olive oil phenols protect HepG2 cells against oxidative stress. Food and Chemical Toxicology 50, 3752-3758.

Saura-Calixto, F., 2011. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. Journal of Agricultural an Food Chemistry 59, 43-49.

Saura-Calixto, F., Bravo, L., 2001. Dietary fiber associated compounds: chemistry, analysis and nutritional effects of polyphenols. In: *Handbook of Dietary Fiber. Second Edition.* S. Sungsoo Cho & M. Dreher (Eds). Marcel Dekker. pp 404-422.

Scalbert, A., Morand, C., Manach, C., Rémésy, C., 2002. Absorption and metabolism of polyphenols in the gut and impact on health. Biomedicine & Pharmacotherapy 56, 276-282.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. The Journal of Nutrition 130, 2073S-2085S.

Scalbert, A., Manach, C., Morand, C., Remesy, C., Jimenez, L., 2005. Dietary polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition. 45, 287-306.

Schalkwijk, C.G., Stehouwer, C.D., 2005. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clinical Science 109, 143-159.

Schenker, S., Heinemann, C., Huber, M., Pompizzi, R., Perren, R., Escher, R., 2002. Impact of roasting conditions on the formation of aroma compounds in coffee beans. Journal of Food Science 67, 60-66.

Schewe, T., Steffen, Y., Sies, H. 2008. How do dietary flavanols improve vascular function? A position paper. Archives of Biochemistry and Biophysics 476,102-106.

Schinella, G., Fantinelli, J.C., Mosca, S.M., 2005. Cardioprotective effects of *Ilex paraguariensis* extract: evidence for a nitric oxide-dependent mechanism. Clinical Nutrition 24, 360-366.

Schinella, G., Fantinelli, J.C., Tournier, H., Prieto, J.M., Spegazzini, E., Debenedetti, S., Mosca, S. M., 2009. Antioxidant and cardioprotective effects of *Ilex brasiliensis*: A comparative study with *Ilex paraguariensis* (yerba mate). Food Research International 42, 1403-1409.

Schinella, G., Neyret, E., Console, G., Tournier, H., Prieto, J. M., Rios, J. L., Giner, R. M., 2014. An aqueous extract of *Ilex paraguariensis* reduces carrageenan-induced edema and inhibits the expression of cyclooxygenase-2 and inducible nitric oxide synthase in animal models of inflammation. Planta Medica 80, 961-968.

Schinella, G.R., Troiani, G., Dávila, V., de Buschiazzo, P.M., Tournier, H.A., 2000. Antioxidant effects of an aqueous extract of *Ilex paraguariensis.* Biochemical and Biophysical Research Communications 269, 357-360.

Schmit, S.L., Rennert, H.S., Rennert, G., Gruber, S.B., 2016. Coffee consumption and the risk of colorectal cancer. Cancer Epidemiology, Biomarkers & Prevention 25, 634-639.

Schneider, Y., Vincent, F., Duranton, B., Badolo, L., Gosse, F., Bergmann, C., Seiler, N., Raul, F., 2000. Anti-proliferative effect of resveratrol, a natural

component of grapes and wine, on human colonic cancer cells. Cancer Letter 158, 85-91.

Schubert, A., Pereira, D. F., Zanin, F. F., Alves, S. H., Beck, R. C. R., Athayde, M. L., 2007. Comparison of antioxidant activities and total polyphenolic and methylxanthine contents between the unripe fruit and leaves of *Ilex paraguariensis* A. St. Hil. Die Pharmazie-An International Journal of Pharmaceutical Sciences 62, 876-880.

Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y., Zhang, Y., Nair, M.G., Heber, D., 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. Journal of Nutritional Biochemistry 16, 360-367.

Sewram, V., De Stefani, E., Brennan, P., Boffetta, P., 2003. Mate consumption and the risk of squamous cell esophageal cancer in Uruguay. Cancer Epidemiology, Biomarkers & Prevention 12, 508-513.

Shahidi, F., Zhong, Y., 2007. Measurement of antioxidant activity in food and biological systems. In: *Antioxidant Measurement and Applications*; Shahidi, F.; Ho, C.-T., Eds.; Oxford University Press: Washington. 36-66.

Shahrzad, S., Bitsch, I., 1998. Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 705, 87-95.

Shan, J.Z., Xuan, Y.Y., Zheng, S., Dong, Q., Zhang, S.Z., 2009. Ursolic acid inhibits proliferation and induces apoptosis of HT-29 colon cancer cells by inhibiting the EGFR/MAPK pathway. Journal of Zhejiang University Science B 10, 668-674.

Shi, X., Dalal, N.S., Jain, A.C., 1991. Antioxidant behaviour of caffeine: efficient scavenging of hydroxyl radicals. Food and Chemical Toxicology 29, 1-6.

Shimazu, T., Inoue, M., Sasazuki, S., Iwasaki, M., Kurahashi, N., Yamaji, T., Tsugane, S., 2008. Coffee consumption and risk of endometrial cancer: a prospective study in Japan. International Journal of Cancer 123, 2406-2410.

Shimazu, T., Tsubono, Y., Kuriyama, S., Ohmori, K., Koizumi, Y., Nishino, Y., Shibuya, D., Tsuji, I., 2005. Coffee consumption and the risk of primary liver cancer: pooled analysis of two prospective studies in Japan. International Journal of Cancer 116, 150-154.

Shimoda, H., Seki, E., Aitani, M., 2006. Inhibitory effect of green coffee bean extract on fat accumulation and body weight gain in mice. BMC Complementary and Alternative Medicine 6, 1-8.

Shrikhande, A.J., 2000. Wine by-products with health benefits. Food Research International 33, 469-474.

Sichieri, R., Everhart, J.E., Mendonça, G.A., 1996. Diet and mortality from common cancers in Brazil: an ecological study. Cadernos da Saude Publica 12, 53-59.

Siegel, R., Ma, J., Zou, Z., Jemal, A., 2014. Cancer statistics. CA: A Cancer Journal for Clinicians 64, 9-29.

Silva, J.F., Bidinotto, L.T., Futardo, K.S., Salvadori, D.M., Rivelli, D.P., Barros, S.B., Rodrigues, M.A., Barbisan, L.F., 2009. Mate attenuates DNA damage and carcinogenesis induced by diethylnitrosamine and thermal injury in rat esophagus. Food and Chemical Toxicology 47, 1521-1529.

Song, Y., Manson, J.A.E., Buring, J.E., Sesso, H.D., Liu, S., 2005. Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: a prospective study and cross-sectional analysis. Journal of the American College of Nutrition 24, 376-384.

Souquet, J.-M., Cheynier, V., Brossaud, F., Moutounet, M., 1996. Polymeric proanthocyanidins from grape skins. Phytochemistry 43, 509-512.

Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., Steiling, H., Williamson, G., Crozier, A., 2009. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. Drug Metabolism and Disposition: the Biological Fate of Chemicals 37, 1749-1758.

Stalmach, A., Steiling, H., Williamson, G., Crozier, A., 2010. Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. Archives of Biochemistry and Biophysics 501, 98-105.

Steinmetz, K.A., Potter, J.D., 1996. Vegetables, fruit, and cancer prevention: a review. Journal of American Dietetic Association 96, 1027-1039.

Stein, J.H., Keevil, J.G., Wiebe, D.A., Aeschlimann, S., Folts, J.D., 1999. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. Circulation 100, 1050-1055.

Stoner, G.D., Mukhtar, H., 1995. Polyphenols as cancer chemopreventive agents. Journal of Cellular Biochemistry S22, 169-180.

Subramanian, A.P., John, A.A., Vellayappan, M.V., Balaji, A., Jaganathan, S. K., Supriyanto, E., Yusof, M., 2015. Gallic acid: prospects and molecular mechanisms of its anticancer activity. Royal Society of Chemistry Advances 5, 35608-35621.

Suhr, Y.-J., 2003. Cancer chemoprevention with dietary phytochemicals. Nature Reviews 3, 768-780.

Sun, J., Li, Y.-Z., Ding, Y.-H., Wang, J., Geng, J., Yang, H., Ren, J., Tang, J.-Y., Gao, J., 2014. Neuroprotective effects of gallic acid against hypoxia/reoxygenation-induced mitochondrial dysfunctions in vitro and cerebral ischemia/reperfusion injury in vivo. Brain Research 1589, 126-139.

Sun, T., Chen, Q.Y., Wu, L.J., Yao, X.M., Sun, X.J., 2012. Antitumor and antimetastatic activities of grape skin polyphenols in a murine model of breast cancer. Food and Chemical Toxicology 50, 3462-3467.

Suzuki, A., Kagawa, D., Ochiai, R., Tokimitsu, I., Saito, I., 2002. Green coffee bean extract and its metabolites have a hypotensive effect in spontaneously hypertensive rats. Hypertension Research 25, 99-107.

Szkudelska, K., Nogowski, L., Szkudelski, T., 2009. Resveratrol, a naturally occurring diphenolic compound, affects lipogenesis, lipolysis and the antilipolytic action of insulin in isolated rat adipocytes. Journal of Steroid Biochemistry and Molecular Biology 113, 17-24.

Szymanska, K., Matos, E., Hung, R.J., Wünsch-Filho, V., Eluf-Neto, J., Menezes, A., Daudt, A.W., Brennan, P., Boffetta, P., 2010. Drinking of mate and the risk of cancers of the upper aerodigestive tract in Latin America: a case-control study. Cancer Causes and Control 21, 1799-1806.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., Conte, A., 2010. In vitro bio-accessibility and antioxidant activity of grape polyphenols. Food Chemistry 120, 599-606.

Taguchi, C., Fukushima, Y., Kishimoto, Y., Suzuki-Sugihara, N., Saita, E., Takahashi, Y., Kondo, K., 2015. Estimated dietary polyphenol intake and major food and beverage sources among elderly japanese. Nutrients 7, 10269-10281.

Tang, N., Wu, Y., Ma, J., Wang, B., Yu, R., 2010. Coffee consumption and risk of lung cancer: a meta-analysis. Lung Cancer 67, 17-22.

Tang, N., Zhou, B., Wang, B., Yu, R., 2009. Coffee consumption and risk of breast cancer: a meta-analysis. American Journal of Obstetric & Gynecology 200, 290-299.

Tarride, J.E., Lim, M., DesMeules, M., Luo, W., Burke, N., O'Reilly, D., Bowen, J., Goeree, R., 2009. A review of the cost of cardiovascular disease. Canadian Journal of Cardiology 25, 195-202.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D.A., Garcia-Viguera, C., 2014. Natural bioactive compounds from winery by-products as health promoters: a review. International Journal of Molecular Sciences 15, 15638-15678.

Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J., Arola, L., Blay, M., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. Journal of Nutritional Biochemistry 20, 210-218.

Tesic, D., Keller, M., Hutton, R.J., 2007. Influence of vineyard floor management practices on grapevine vegetative growth, yield, and fruit composition. American Journal of Enology and Viticulture 58, 1-11.

Thimothe, J., Bonsi, I.A., Padilla-Zakour, O.I., Koo, H.. 2007. Chemical characterization of red wine grape (*Vitis vinifera* and *Vitis* interspecific hybrids)

and pomace phenolic extracts and their biological activity against *Streptococcus mutans*. Journal of Agricultural and Food Chemistry 55, 10200–10207.

Thom, E., 2007. The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. Journal of International Medical Research 35, 900-908.

Tomás-Barberán, F.A., Andrés-Lacueva, C., 2012. Polyphenols and health: current state and progress. Journal of Agricultural and Food Chemistry 60, 8773-8775.

Tomás-Barberán, F.A., Clifford, M.N., 2000. Flavanones, chalcones and dihydrochalcones. Nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture 80, 1073-1080.

Tomás-Barberán, F.A., García-Villalba, R., González-Sarrías, A., Selma, M.V., Espín, J.C., 2014. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. Journal of Agricultural and Food Chemistry 62, 6535-6538.

Torres, J.L., Bobet, R., 2001. New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio−flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. Journal of Agricutural and Food Chemistry 49, 4627-4634.

Tresserra-Rimbau, A., Medina-Remon, A., Perez-Jimenez, J., Martinez-Gonzalez, M.A., Covas, M.I., Corella, D., Salas-Salvado, J., Gomez-Gracia, E., Lapetra, J., Aros, F., Fiol, M., Ros, E., Serra-Majem, L., Pinto, X., Munoz, M.A., Saez, G.T., Ruiz-Gutierrez, V., Warnberg, J., Estruch, R., Lamuela-Raventos, R.M., 2013. Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: the PREDIMED study. Nutrition Metabolism & Cardiovascular Diseases 23, 953-959.

Tribolo, S., Lodi, F., Winterbone, M.S., Saha, S., Needs, P.W., Hughes, D.A., Kroon, P.A., 2013. Human metabolic transformation of quercetin blocks its capacity to decrease endothelial nitric oxide synthase (eNOS) expression and

endothelin-1 secretion by human endothelial cells. Journal of Agricultural and Food Chemistry 61, 8589-8596.

Trugo, L.C., Macrae, R., 1984. A study of the effect of roasting on the chlorogenic acid composition of coffee using HPLC. Food Chemistry 15, 219-227.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2, 1231-1246.

Tseng, A., Zhao, Y., 2012. Effect of different drying methods and storage time on the retention of bioactive compounds and antibacterial activity of wine grape pomace (Pinot Noir and Merlot). Journal of Food Science 77, 192-201.

Tsuda, T., Ueno, Y., Aoki, H., Koda, T., Horio, F., Takahashi, N., Kawada, T., Osawa, T., 2004. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. Biochemical and Biophysical Research Communications 316, 149-157.

Tung, Y. T., Wu, J. H., Huang, C. C., Peng, H. C., Chen, Y. L., Yang, S. C., Chang, S. T., 2009. Protective effect of *Acacia confusa* bark extract and its active compound gallic acid against carbon tetrachloride-induced chronic liver injury in rats. Food and Chemical Toxicology 47, 1385-1392.

Tunnicliffe, J.M., Shearer, J., 2008. Coffee, glucose homeostasis, and insulin resistance: physiological mechanisms and mediators. Applied Physiology, Nutrition, and Metabolism 33, 1290-1300.

Turati, F., Galeone, C., Edefonti, V., Ferraroni, M., Lagiou, P., La Vecchia, C., Tavani, A., 2012. A meta-analysis of coffee consumption and pancreatic cancer. Annals of Oncology 23, 311-318.

Vaidyanathan, J.B., Walle, T., 2003. Cellular uptake and efflux of the tea flavonoid (-) epicatechin-3-gallate in the human intestinal cell line Caco-2. The Journal of Pharmacology and Experimental Therapeutics 307, 745-752.

Van Dam, R.M., Feskens, E.J.M., 2002. Coffee consumption and risk of type 2 diabetes mellitus. The Lancet 360, 1477-1478.

Van Rooij, J., van der, Stegen G.H., Schoemaker, R.C., Kroon, C., Burggraaf, J., Hollaar, L., Vroon, T.F., Smelt, A.H., Cohen, A.F., 1995. A placebo-controlled parallel study of the effect of two types of coffee oil on serum lipids and

transaminases: identification of chemical substances involved in the cholesterol-raising effect of coffee.The American Journal of Clinical Nutrition 61, 1277-1283.

Venkatesan, T., Choi, Y.-W., Mun, S.-P., Kim, Y.-K., 2016. *Pinus radiata* bark extract induces caspase-independent apoptosis-like cell death in MCF-7 human breast cancer cells. Cell Biology and Toxicology 32, 451-464.

Vinson, J.A., Burnham, B.R., Nagendran, M.V., 2012. Randomized, double-blind, placebo-controlled, linear dose, crossover study to evaluate the efficacy and safety of a green coffee bean extract in overweight subjects. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy 5, 21-27.

Vita, J., 2005. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. The American Journal of Clinical Nutrition 81, 292S-297S.

Vogiatzoglou, A., Mulligan, A.A., Lentjes, M.A.H., Luben, R.N., Spencer, J.P.E., Schroeter, H., Khaw, K.-T., Kuhnle, G.G.C., 2015. Flavonoid intake in European adults (18 to 64 years). PLoS ONE 10, e0128132.

Wallerath, T., Li, H., Godtel-Ambrust, U., Schwarz, P.M., Forstermann, U., 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 12, 97-104.

Williamson, G., Manach, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. The American Journal of Clinical Nutrition 81, 243S-255S.

Wang, J., Tang, L., Wang, J.S., 2015. Biomarkers of dietary polyphenols in cancer studies: current evidence and beyond. Oxiditive Medicine and Cellular Longevity 2015, 732302-732316.

Wang, S., Mateos, R., Goya, L., Amigo-Benavent, M., Sarriá, B., Bravo, L., 2016. A phenolic extract from grape by-products and its main hydroxybenzoic acids protect Caco-2 cells against pro-oxidant induced toxicity. Food and Chemical Toxicology 88, 65-74.

Wang, Y., Ding, L., Wang, X., Zhang, J., Han, W., Feng, L., Sun, J., Jin, H., Wang, X.J., 2012. Pterostilbene simultaneously indices apoptosis, cell cycle arrest and cyto-protective autophagy in breast cancer cells. American Journal of Translational Research 4, 44-51.

Wang, Z. M., Gao, W., Wang, H., Zhao, D., Nie, Z. L., Shi, J. Q., Zhao, S., Lu, X., Wang, L. S., Yang, Z. J., 2014. Green tea polyphenol epigallocatechin-3-gallate inhibits TNF-alpha-induced production of monocyte chemoattractant protein-1 in human umbilical vein endothelial cells. Cellular Physiology and Biochemistry 33, 1349-1358.

Watanabe, T., Arai, Y., Mitsui, Y., Kusaura, T., Okawa, W., Kajihara, Y., Saito, I., 2006. The blood pressure-lowering effect and safety of chlorogenic acid from green coffee bean extract in essential hypertension. Clinical and Experimental Hypertension 28, 439-449.

Waterhouse, A.L., 2002. Wine phenolics. Annals of the New York Academy of Sciences 957, 21-36.

Wedick, N.M., Pan, A., Cassidy, A., Rimm, E.B., Sampson, L., Rosner, B., Willett, W., Hu, F.B., Sun, Q., van Dam, R.M., 2012. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. The American Journal of Clinical Nutrition 95, 925-933.

Welch, C.R., Wu, Q., Simon, J.E., 2008. Recent advances in anthocyanin analysis and characterization. Current Analytical Chemistry 4, 75-101.

Welder, A.A., Acosta, D., 1994. Enzyme leakage as an indicator of cytotoxicity in cultured cells. In: *Methods in Toxicology*, Volume 1 Part B, In Vitro Toxicology Indicators. Tyson, C.A., Frazier, J.M. (Eds.), Academic Press, San Diego, 46-49.

WHO, 1948. Preamble to the Constitution of the World Health Organization as adopted by the International Health Conference, New York, 19-22 June, 1946. Official Records of the World Health Organization 2, 100.

Widlansky, M.E., Gokce, N., Keaney, J.F.Jr., Vita, J.A., 2003. The clinical implications of endothelial dysfunction. Journal of the American College of Cardioloy 42, 1149-1160.

Wightman, J.D., Heuberger, R.A., 2015. Effect of grape and other berries on cardiovascular health. Journal of the Science of Food and Agriculture 95, 1584-1597.

Wnuk, M., Lewinska, A., Oklejewicz, B., Bugno, M., Slota, E., Bartosz, G., 2009. Evaluation of the cyto- and genotoxic activity of yerba mate (*Ilex paraguariensis*) in human lymphocytes in vitro. Mutation Research 679, 18-23.

Wolfram, S., Raederstorff, D., Wang, Y., Teixeira, S.R., Elste, V., Weber, P., 2005. TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass. Annals of Nutrition and Metabolism 49, 54-63.

Wu, W., Tong, Y., Zhao, Q., Yu, G., Wei, X., Lu, Q., 2015. Coffee consumption and bladder cancer: a meta-analysis of observational studies. Nature Scientific Reports 5, article 9051, 1-13.

Xia, E.Q., Deng, G.F., Guo, Y.J., Li, H.B., 2010. Biological activities of polyphenols from grapes. International Journal of Molecuelar Sciences 11, 622-646.

Xiao, J.B., Hogger, P., 2015. Dietary polyphenols and type 2 diabetes: current insights and future perspectives. Current Medicine Chemistry 22, 23-38.

Xie, Y., Qin, J., Nan, G., Huang, S., Wang, Z., Su, Y., 2016. Coffee consumption and the risk of lung cancer: an updated meta-analysis of epidemiological studies. European Journal of Clinical Nutrition 70, 199-206.

Xu, C., Zhang, Y., Cao, L., Lu, J., 2010. Phenolic compounds and antioxidant properties of different grape cultivars grown in China. Food Chemistry 119, 1557-1565.

Xu, D., Perez, R.E., Ekekezie, II, Navarro, A., Truog, W.E., 2008. Epidermal growth factor-like domain 7 protects endothelial cells from hyperoxia-induced cell death. American Physiological Society-Lung Cellular and Molecular Physiology 294, 17-23.

Yamaguchi, T., Chikama, A., Mori, K., Watanabe, T., Shioya, Y., Katsuragi, Y., Tokimitsu, I., 2008. Hydroxyhydroquinone-free coffee: a double-blind, randomized controlled dose-response study of blood pressure. Nutrition, Metabolism and Cardiovascular Diseases 18, 408-414.

Yamashita, K., Yatsuya, H., Muramatsu, T., Toyoshima, H., Murohara, T., & Tamakoshi, K., 2012. Association of coffee consumption with serum adiponectin,

leptin, inflammation and metabolic markers in Japanese workers: a cross-sectional study. Nutrition and Diabetes 2, e33-e38.

Yamashita, S., Furubayashi, T., Kataoka, M., Sakane, T., Sezaki, H., Tokuda, H., 2000. Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. European Journal of Pharmaceutical Sciences 10, 195-204.

Yang, S., Zhang, H., Yang, X., Zhu, Y., Zhang, M., 2015. Evaluation of antioxidative and antitumor activities of extracted flavonoids from Pink Lady apples in human colon and breast cancer cell lines. Food & Function 6, 3789-3798.

Yen, G. C., Chen, Y. C., Chang, W. T., Hsu, C. L., 2011. Effects of polyphenolic compounds on tumor necrosis factor-alpha (TNF-alpha)-induced changes of adipokines and oxidative stress in 3T3-L1 adipocytes. Journal of Agricultural and Food Chemistry 59, 546-551.

Yilmaz, Y., Toledo, R.T., 2004. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. Journal of Agricultural and Food Chemistry 52, 255-260.

Yi, W., Fischer, J., Akoh, C.C., 2005. Study of anticancer activities of muscadine grape phenolics *in vitro*. Journal of Agricultural and Food Chemistry 53, 8804-8812.

Yochum, L., Kushi, L.H., Meyer, K., Folsom, A.R., 1999. Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. American Journal of Epidemiology 149, 943-949.

Yonguc, G. N., Dodurga, Y., Adiguzel, E., Gundogdu, G., Kucukatay, V., Ozbal, S., Yilmaz, I., Cankurt, U., Yilmaz, Y., Akdogan, I., 2015. Grape seed extract has superior beneficial effects than vitamin E on oxidative stress and apoptosis in the hippocampus of streptozotocin induced diabetic rats. Gene 555, 119-126.

Yoshimoto, M., Yahara, S., Okuno, S., Islam, M.S., Ishiguro, K., Yamakawa, O., 2002. Antimutagenicity of mono, di, and tricaffeoylquinic acid derivatives isolated from sweetpotatoe (*Ipomoea batatas* L.) leaf. Bioscience, Biotechnology, and Biochemistry 66, 2336-2341.

Yu, J., Ahmedna, M., 2013. Functional components of grape pomace: their composition, biological properties and potential applications. International Journal of Food Science & Technology 48, 221-237.

Yu, S., Yue, S., Liu, Z., Zhang, T., Xiang, N., Fu, H., 2015. Yerba mate (*Ilex paraguariensis*) improves microcirculation of volunteers with high blood viscosity: a randomized, double-blind, placebo-controlled trial. Experimental Gerontology 62, 14-22.

Zamora-Ros, R., Luján-Barroso, L., Bueno de Mesquita, H.B., Dik, V.K., Boeing, H., Steffen, A., Tjornneland, A., Olsen, A., Bech, B.H., Overvad, K., Boutron-Ruault, M.-C., Racine, A., Fagherazzi, G., Kuhn, T., Katzke, V., Trichopoulou, A., Lagiou, P., Trichopoulos, D., Tumino, R., Panico, S., Vineis, P., Grioni, S., Palli, D., Weiderpass, E., Skeie, G., Huerta, J.M., Sanchez, M.-J., Argüelles, M., Amiano, P., Ardanaz, E., Nilsson, L., Wallner, B., Lindkvist, B., Wallström, P., Peeters, P.H.M., Key, T.J., Khaw, K.-T., Wareham, N.J., Freisling, H., Stepien, M., Ferrari, P., Gunter, M.J., Murphy, N., Riboli, E., Gonzalez, C.A., 2014. Tea and coffee consumption and risk of esophageal cancer: the European prospective investigation into cancer and nutrition study. International Journal of Cancer 135, 1470-1479.

Zapaterini, J.R, Bidinotto, L.T., Rodrigues, M.A.M., Barbisan, L.F., 2010. Chemopreventive effects of mate against mouse mammary and colon carcinogenesis. Human & Experimental Toxicology 29, 175-185.